PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12N 7/00, C12Q 1/68, 1/70
C07K 15/02, 15/12, A01N 63/00

A1

(11) International Publication Number: WO 94/00562

(43) International Publication Date: 6 January 1994 (06.01.94)

(21) International Application Number: PCT/US93/06162

(22) International Filing Date: 23 June 1993 (23.06.93)

(30) Priority data:
903,421 24 June 1992 (24.06.92) US

(71) Applicants: THE MT. SINAI SCHOOL OF MEDICINE OF THE CITY UNIVERSITY OF NEW YORK [US/US]; One Gustave Levy Place, New York, NY 10029 (US). CORNELL RESEARCH FOUNDATION, INC. [US/US]; 20 Thornwood Drive, Suite 105, Ithaca, NY 14850 (US).

(72) Inventors: GELMAN, Irwin, H. ; 501 West 110th Street, New York, NY 10025 (US). LAURENCE, Jeffrey, C. ; 3 Briar Close, Larchmont, NY 10538 (US).

(74) Agents: CORUZZI, Laura, A. et al.; Pennie & Edmonds, 1155 Avenue of the Americas, New York, NY 10036 (US).

(81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: A NOVEL HUMAN IMMUNODEFICIENCY VIRUS

(57) Abstract

The present invention relates to a new variety of retroviruses distinct from HIV-1 and HIV-2, designated HIV-LP. The isolation, characterization and cloning of a prototype HIV-LP is described. The invention also relates to nucleotide sequences derived from the HIV-LP family, viral proteins and antigens and antibodies specific for HIV-LP which can be used for diagnostic and/or therapeutic purposes.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NE	Niger
BE	Belgium	GN	Guinea	NL	Netherlands
BF	Burkina Faso	GR	Greece	NO	Norway
BG	Bulgaria	HU	Hungary	NZ	New Zealand
BJ	Benin	IE	Ireland	PL	Poland
BR	Brazil	IT	Italy	PT	Portugal
BY	Belarus	JP	Japan	RO	Romania
CA	Canada	KP	Democratic People's Republic of Korea	RU	Russian Federation
CF	Central African Republic			SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovak Republic
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	UA	Ukraine
DE	Germany	MG	Madagascar	US	United States of America
DK	Denmark	ML	Mali	UZ	Uzbekistan
ES	Spain	MN	Mongolia	VN	Viet Nam
FI	Finland				

A NOVEL HUMAN IMMUNODEFICIENCY VIRUS

1. INTRODUCTION

The present invention relates to a new variety of retroviruses distinct from HIV-1 and HIV-2, designated HIV-LP. The isolation, characterization and cloning of a prototype HIV-LP is described. The invention also relates to nucleotide sequences derived from the HIV-LP family, viral proteins and antigens and antibodies specific for HIV-LP which can be used for diagnostic and/or therapeutic purposes.

2. BACKGROUND OF THE INVENTION

Acquired immune deficiency syndrome (AIDS) is etiologically linked to two subtypes of human immunodeficiency virus, HIV-1,-2. However, in certain acquired cellular immune defects among patients with risk factors for HIV neither HIV-1 or -2 can be detected. These encompass homosexual men with aggressive Kaposi's sarcoma and varying degrees of cellular immune deficiency who are negative for HIV-1,-2 by serology, viral cultures and DNA amplification by polymerase chain reaction (PCR) (A.E. Friedman-Kien, et al., 1990, Lancet 335: 168; V. Soriano et al., VII Intl. Conf. AIDS, Florence, Italy, June 16-21, 1991, Abst. TuB82), as well as a recent case report of a young homosexual male with profound CD4+ T-cell depletion, Kaposi's sarcoma, Pneumocystis carinii pneumonia, and similar lack of evidence for HIV-1,-2 infection (B. Safai, et al., VII Intl. Conf. AIDS, Florence, Italy, June 16-21, 1991, Abst. TuB83). It would be highly desirable to identify the agent or agents responsible for the clinical symptoms in such patients, and to devise diagnostic assays that can be used to test blood supplies for the presence of the

infectious agent. However, heretofore, this agent had not been identified.

3. SUMMARY OF THE INVENTION

A new variant of the human immunodeficiency virus, distinct from HIV-1 or HIV-2 is described. This new member of the human lentivirus family, referred to herein as HIV-LP, can be used to construct clones, engineer nucleotide sequences, polypeptides, and antigens useful for diagnosis and/or therapy. The invention is based, in part, on the discovery of the prototype of this new member of the human lentivirus family, in individuals having clinical symptoms associated with ARC (AIDS related complex) or AIDS, but who are negative for HIV-1,-2 as determined by standard immunoassay, PCR analysis and cell culture/p24 HIV-1,-2 Gag antigen assays. In particular, three individuals with profound CD4+ T lymphocyte depletion and opportunistic infections were identified, two of whom had known AIDS risk factors. There was no evidence for HIV-1,-2 infection by serology, DNA amplification by high stringency polymerase chain reaction (PCR), and co-culture with assay for p24 Gag. However, particulate RNA directed DNA polymerase activity was observed in cultures from two available individuals, and low-stringency PCR amplification of HIV-1 tat, pol, and/or HIV-1,-2 gag sequences gave signals of the predicted sizes with all patient samples. One isolate, selected for further study, could infect mitogen-activated peripheral blood lymphocytes in a CD4-restricted manner. Immunoprecipitation of [^{35}S]-labeled proteins from infected cells using patient antisera yielded bands of molecular weight 140, 41 and 27 kD, representing the putative envelope, transmembrane and core molecules of HIV-LP, respectively. cDNA was

synthesized from purified virions, and recombinant plasmids were constructed, cloned and sequenced providing partial sequences of env, LTR/nef, and pol with significant homology to HIV. This agent, termed "HIV-LP," is apparently a new variant of the HIV family, and may be of significant public health concern.

This invention relates to the family of HIV-LP viruses, nucleotide sequences derived from HIV-LP viruses, viral proteins and viral antigens, and antibodies specific for HIV-LP which can be used for a variety of ends including diagnostics and therapeutics.

4. DESCRIPTION OF THE FIGURES

FIG. 1. Reverse transcriptase (RT) activity and HIV Gag antigen in supernatants of Pt. 1 and Pt. 2 PBMC cultures. 2 x 10^6/ml PBMC from Pt. 1 (A) or an HIV-1 seropositive control (B) were incubated for 3 days with 5 µg/ml PHA-P (Sigma) in RPMI 1640 plus 10% fetal bovine serum, then resuspended at 0.5 x 10^6/ml in culture medium with 32 U/ml IL-2 (Electro-Nucleonics, Fairfield, NJ) in a total volume of 2 ml in polyvinyl flat-bottom macrowells (Falcon B-D Labware, Lincoln Pk, NJ). Co-cultures were performed by incubating 0.5 x 10^6 Pt. 1 (C) or Pt. 2 (D) PBMCs with 0.5 x 10^6 normal donor PBMCs, pre-activated for 48-72 hours with PHA, in 2 ml of culture medium plus IL-2. Cell-free transmission of RT activity was demonstrated by exposure of PHA-activated PBMC cultures from two different donors (E, F) to 2 x 10^3 counts/minute of RT activity from Pt. 1, together with 2 µg/ml of the anionic resin polybrene (Sigma). The medium was completely changed 18 hours later. For each culture, one-half of the supernatant was removed every 3-4 days, evaluated for RT activity and p24 Ag, and replenished

with fresh medium plus IL-2. RT assays were performed as previously described (Laurence et al., 1987, Science 235: 1501). p24 Ag was detected by ELISA (Abbott Labs, Chicago, IL) using rabbit Ig directed against HIV-1 epitopes and contained in polystyrene beads, as per the manufacturer's directions. It has a sensitivity of ≥ 30 pg/ml.

FIG. 2. Multi-nucleated giant cells induced by Pt. 1 RT+ supernatants in PHA-activated normal donor PBMCs (upper photo) and a clone of CD4+ HUT-78 T-cells (lower photo).

FIG. 3. A: Immunoblots of HIV-1 antigens with various sera. Nitrocellulose strips containing HIV-1 antigens were incubated with 1:40 dilutions of sera, then developed with alkaline phosphatase-conjugated anti-human IgG and colorimetric reagents, as per the manufacturer's directions (DuPont, Wilmington, DE). Lane 1, HIV-1 seropositive donor. Lane 2, HIV-1,-2 seronegative donor without AIDS risk factors. Lane 3, Pt. 1. Lane 4, PT. 2.

B: Radioimmunoprecipitation of [^{35}S]cysteine-labeled lysates of an isolate from Pt. 1. PHA-activated donor PBMC exposed to Pt. 1 RT activity were harvested at the peak of RT production, labeled for 16 hours, and cellular lysates precipitated with 5 μl aliquots of various sera, as previously detailed (Laurence et al., 1987, Science 235: 1501; Laurence et al., 1990, Cell. Immunol. 128: 337). Lane 1, molecular weight markers. Lane 2, HIV-1,-2 seronegative donor without AIDS risk factors. Lane 3, HIV-1 seropositive donor (pre-selected, based on ELISA reactivity with Pt. 1 PBMC lysates, from six others showing no such reactivity). Lane 4, HIV-2 seropositive donor. Lane 5, Pt. 1. Arrows indicate areas of specific reactivity with Pt. 1 serum.

FIG. 4. Thin-section electron photomicrographs of PHA-activated normal donor PBMC inoculated with RT+ Pt. 1 supernatant. Cells were fixed in Karnofsky solution (2.5% glutaraldehyde, 0.5% paraformaldehyde, 0.1M phosphate buffer, pH 7.2) for 2 days at 4°C, post-fixed in 1% OsO_4, plastic embedded, sectioned, and treated with 0.5% lead acetate. Lymphoblasts are magnified 31,000 X. The insert shows a particle with an electron-dense internal core (100,000 X).

FIG. 5. PCR analysis for HIV-1 tat and HIV-1,-2 gag. DNA was extracted from PBMC co-cultures (Pt. 1) using an anionic chromatography column (A.S.A.P., Boehringer-Mannheim, Indianapolis, IN), as per manufacturer's instructions, or from paraffin sections of an intestinal lamina propria biopsy (Pt. 3), using published procedures (Wright & Manos, In PCR Protocols: A Guide to Methods and Applications (Academic Press, NY, 1990), p. 153). High stringency involved 2.5 U Taq polymerase (Perkin-Elmer-Cetus, Norwalk, CT), 0.75 mM $MgCl_2$, 200 μM of oligonucleotide primers, and 0.2 μg of DNA in a total volume of 50 μl. Samples were overlaid with 50 μl mineral oil, preheated to 80°C for 8 minutes, then 90°C for 45 seconds, followed by injection of deoxynucleoside triphosphates (dNTP; 200 μM final concentration). DNA was amplified for 40 cycles at 94°C for 1 minute, 55°C for 2 minutes, and 72°C for 2 minutes. Low stringency conditions were similar except that greater amounts of $MgCl_2$ (1.5 mM) and Taq (3.75 U) were included, and the annealing temperature was lowered to 42°C. Controls included U1.1, a human promonocytic cell line that contains two integrated copies of HIV-1 per cell (Folks, et al., 1988, J. Immunol. 140: 1117), reaction mixture without added sample ("no DNA"), and DNA extracted from PHA-

activated PBMCs derived from a healthy, HIV-1 seronegative donor ("control"). The integrity of all DNAs was evaluated by amplification for β-globin. 20 μl of each sample was electrophoresed through a 1.5% agarose minigel containing 0.5 mg/ml ethidium bromide, then photographed under UV illumination. Primers: HIV-1 tat, designed to amplify a 171 bp segment from position 5359 to 5529, ACAGAGGAGAGCAAGAAATGG (sense) and GCTTCTTCCTGCCATAGG (anti-sense); HIV-1,-2 gag (Perkin-Elmer-Cetus), designed to amplify a 142 bp segment from position 1366 to 1507 of HIV-1, AGTGGGGGGACATCAAGCAGCCATGCAAAT (sense) and TGCTATGTCAGTTCCCCTTGGTTCTCT (anti-sense); β-globin (Perkin-Elmer-Cetus), designed to amplify a 268 bp fragment, CAACTTCATCCACGTTCACC (sense) and GAAGAGCCAAGGACAGGTAC (anti-sense).

FIG. 6. Molecular hybridizations with Pt. 1 isolate. Virions were purified from 90 ml of supernatant from a co-culture of Pt. 1 and normal donor PHA-activated PBMCs, ultra-centrifuged, and aliquots used for hybridization and cloning. A,B: Pellets resuspended in TNE buffer (10 mM Tris-HCl, pH 7.4, 0.1 M NaCl, 1 mM EDTA) with 0.1% SDS were spotted onto nitrocellulose strips presoaked in 2X SSC (standard saline citrate; 0.3 M NaCl, 0.03 M sodium citrate), baked, and hybridized for 16 hours under conditions of high (45% formamide, 6X SSC, 68°C) or low (30% formamide, 5X SSC, 42°C) stringency, then washed in 2X SSC with 0.1% SSD at 68°C or 42°C, respectively. Probes represent HIV-1 env, tat, LTR (A) and tat-nef/LTR (B) sequences in linearized pBR322 plasmids, illustrated elsewhere (Laurence et al., 1987, J. Clin. Invest. 80: 1631). Procedures for probe labeling with digoxigenin-11-dUTP by the random primer method, and DNA detection using a polyclonal sheep anti-

digoxigenin-Fab' of IgG conjugated with alkaline phosphatase have been previously published (Laurence et al., 1990, Blood 75: 696).

FIG. 7. Nucleotide sequences of Pt. 1 isolate. A cDNA first strand using oligo(dT) (Dynabeads dT_{25}, Dynal, Great Neck, NY) was synthesized from Pt. 1 pellet using MLV RT as described (Clavel et al., 1986, Nature 324: 691). Product was tailed with dCTP by terminal deoxynucleotide transferase, then converted into dsDNA by the DNA polymerase I/RNase H method (GIBCO-BRL, Bethesda, MD). This cDNA was amplified by PCR for 30 cycles (94°C, 1 minute; 55°C, 2 minutes, 72°C, 3 minutes) using the following primers: $GCGAAAGCTTG_{15}$ and $CGAGGAATTCT_{30}$. Following removal of excess primers by filtration through Sephadex G-50 spin columns, the products were cut with EcoR1 and HindIII and ligated to pBluescript KS (Stratagene, La Jolla, CA). Nucleotide sequencing was performed on (double-stranded) plasmid DNA using a Sequenase 2.0 kit (U.S. Biochemical, Cleveland, OH).

A: Nucleotide sequence from HIV-LP, cDNA clones JS-3 and JS-5 (SEQ. ID NO:1). A stretch of residues identical to a region of HIV-1 strain SF-2 <u>env</u> (nucleotides 6612-6827; Myers et al., eds. Human Retroviruses and AIDS 1991: a compilation and analysis of nucleic acid and amino acid sequences, Los Alamos National Lab., Los Alamos, NM, 1991) are underlined.

B: Nucleotide sequence from HIV-LP cDNA clone JS-8 (SEQ. ID NO:2). Residues identical to a region of HIV-1 HXB2 clone LTR/<u>nef</u> (nucleotides 718-579; Myers et al., eds. Human Retroviruses and AIDS 1991: a compilation and analysis of nucleic acid and amino acid sequences, Los Alamos National Lab., Los Alamos, NM, 1991) are underlined.

C: Nucleotide sequence from HIV-LP, cDNA clone JS-2 (SEQ. ID NO:3). Residues identical to a region of HIV-2 (strain ST) pol (nucleotides 3347-3524) are underlined. The similarity between HIV-LP and HIV-2_{ST} in this region is 56%, as compared to a 58% similarity between HIV-2_{ST} and HIV-1_{HXB2}.

5. DETAILED DESCRIPTION OF THE INVENTION

The invention relates to a new variant of the HIV family, referred to herein as HIV-LP. Clones and nucleotide sequences which can be used in diagnostic hybridization assays or for the cloning and expression of viral antigens are also encompassed by the invention. The invention also relates to the production of viral proteins and antigens by recombinant DNA or synthetic chemical techniques. Such antigens may be used in immunoassays for the diagnosis of HIV-LP infection, or in the development of vaccine formulations.

The invention is described in the subsections below and by the examples detailed in Sections 6 and 7, infra, which document the existence, in three individuals residing in New York City, of an acquired cellular immune deficiency associated with opportunistic infections and malignancies pathognomonic of AIDS or AIDS-related complex, occurring in the absence of evidence for HIV-1,-2 infection by serology, viral culture and PCR. Such persistent and progressive CD4+ T-cell depletion is distinctly unusual outside of HIV infection (Bofill, et al., 1992, Clin. Exp. Immunol. 88: 243). For example, it was not seen in a group of elderly HIV-1,-2 seronegative, retroviral culture (p24 Gag and RT) negative individuals with Pneumocystis carinii pneumonia and no HIV risk factors (Jacobs, et al., 1991, N. Engl. J. Med. 324: 246).

A retroviral isolate was partially characterized from Patient no. 1 (Pt. 1). Its CD4 cell tropism, pattern of cytopathic and cytolytic effects, biochemistry of its polymerase, A/C codon usage, and partial sequences of its env, LTR/nef, and pol genes clearly place it in the lentivirus family, with similarities to HIV-1,-2, as well as distinct differences. All HIV-1 and -2 isolates described thus far share common serologic properties, and though there may be substantial variation in nucleic acid and protein composition, many antigenic components immunologically cross-react (Clavel, 1987, AIDS 1: 135). This is clearly not the case for our examples. Pending further sequence and molecular taxonomy data, we suggest calling this subtype and members of its family, HIV-LP.

Given the cross-reactive serologies of Patients 1 and 2, described infra, and similarities in PCR patterns among all three patients, and subject to sequencing the full length provirus, it appears that these patients are infected with the same agent. The public health implications of these findings are clear. Based on the information from these cases and from in vitro studies described infra, HIV-LP is probably transmissible by the same routes as HIV-1,-2, and as reported for HIV-2 (O'Brien, et al., 1992, Amer. Med. Assoc. 267: 2775) may co-infect HIV-1+ individuals, and will certainly escape detection by current serological surveillance mechanisms.

5.1. THE HIV-LP VIRUS

The prototype HIV-LP virus was originally isolated from a patient with clinical AIDS who is negative for both HIV-1 and HIV-2 as determined by immunoassay, PCR (polymerase chain reaction) analysis,

and cell culture/p24 antigen assays. The patient was also negative for human T-cell lymphotropic viruses type I and II (HTLV-I, II). Samples of cells infected with the HIV-LP isolated from this patient have been deposited with the American Type Culture Collection (ATCC) and assigned accession no. VR 2374. The isolation and characterization of this HIV-LP virus isolate is described in Section 6, infra. However, the invention includes HIV-LP strain variants and viruses which are functionally equivalent as described in the subsections below.

5.1.1. ISOLATION AND CHARACTERIZATION OF THE HIV-LP VIRUS

In accordance with the invention, the samples deposited with the ATCC can be used as a source of virus for propagation, cloning, etc. Alternatively, the HIV-LP can be isolated from other patients with clinical ARC or AIDS who are negative for HIV-1,-2 and HTLV-I,II. To this end, the virus can be propagated and expanded by co-culturing PBMCs (peripheral blood mononuclear cells) obtained from the patient with normal PBMCs activated with PHA (phytohemagglutinin) or other appropriate T-cell mitogens. Alternatively, the virus (obtained from patients or the ATCC deposit) may be propagated in continuous CD4+ cell lines including but not limited to HUT-78, for example. The virus can be isolated from the culture supernatants by centrifugation (e.g., at 1000 x g), layered over a discontinuous sucrose gradient and purified by ultra-centrifugation (e.g., 33,000 rpm in a SW41 rotor for 12 hours at 4°C). The HIV-LP sediments at a density between 1.12 to 1.16 g/ml; a density which is characteristic of mammalian retroviruses.

The HIV-LP virus displays a tropism for CD4 positive cells. Immunoprecipitation of viral antigens

from infected cell cultures using patient antisera identifies three viral antigens of molecular weight 130-140, 41 and 27 kD, corresponding to the envelope, transmembrane and core viral proteins.

Preliminary sequence information, obtained by cloning the cDNA synthesized from viral RNA of the deposited HIV-LP is described herein (see Section 6.4.3 and FIG. 7). The cDNA sequences corresponding to HIV-LP env (JS-3, JS-5; FIG. 7A; SEQ. ID NO:1), the LTR including the nef region (JS-8; FIG. 7B; SEQ. ID NO:2), and the pol region (JS-2; FIG. 7C; SEQ. ID NO: 3) are described. Corresponding cDNA clones JS-3, JS-5, JS-2, and JS-8 have also been deposited with the ATCC and assigned accession nos. 69011, 69012, and 69013, respectively.

The viruses of the invention include the HIV-LP virus as deposited with the ATCC, including any related virus strains of HIV-LP and functional equivalents, i.e., retroviruses having equivalent antigenic and immunologic properties. Even though HIV-LP strains may vary genetically rather substantially, the diverse strains should have certain common antigenic sites on the structural proteins, e.g., core, envelope and the transmembrane protein. Therefore, the prototype strain deposited with the ATCC may be used as a source of antigen to detect strain variants and functional equivalents i.e., non-HIV-1,-2 viruses that immunologically cross react with HIV-LP at any level. Strain variants of HIV-LP are defined herein as retroviruses which can be identified as HIV-LP based on at least one of the following criteria: immunoassay or hybridization analysis, including nucleotide amplification techniques (e.g., PCR, LCR), using antibodies, antigens or oligonucleotide probes or

primers designed on the basis of the prototype HIV-LP as deposited with the ATCC and described herein.

5.1.2. CLONING AND SEQUENCING THE VIRAL GENOME

The HIV-LP prototype has been isolated, cultivated in PHA-stimulated co-cultures, and regions of the virus (e.g., env, LTR/nef, and pol) have been subcloned and sequenced as described in Section 5.1.1 and in the working examples described in Section 6, et seq. This material, which has been deposited by the ATCC, can be used to complete the cloning and sequencing of the viral genome using any of a number of techniques which are well known to those skilled in the art, including but not limited to the construction of cDNA libraries corresponding to the HIV-LP genome which can be used to "walk-out" the remaining sequence; RACE (Rapid Amplification of cDNA Ends) which involves the use of full length viral RNA and oligonucleotide primers to amplify cDNA ends to generate a full length cDNA clone(s); individual HIV-LP genes can be cloned from infected cells using oligonucleotide primers in a polymerase amplification assay (e.g. PCR, LCR) designed for the desired gene located within the proviral sequence, e.g., pol, gag, env, etc. For a review of such techniques, see Maniatis et al., 1989, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y.; and Ausubel et al., 1989, Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley Interscience, N.Y.

Alternatively, the protocol described in Section 6, et seq., infra, could be used to clone and sequence HIV-LP isolated from other AIDS/ARC patients who are HIV-1,-2 negative, as determined by both immunoassay and hybridization/amplification assays.

In another embodiment of the invention, an expression cloning approach could be taken (see Maniatis, and Ausubel, supra). In this regard, cDNA copies of the HIV-LP genome could be cloned into an expression library such as λgt 11 and screened using antisera or antibodies derived from AIDS/ARC patients who are HIV-1,-2 negative as determined by both immunoassay and hybridization/amplification assays. Since serum antibodies specific for authentic viral antigens responsible for the immune response generated in an infected individual are used to screen the library, this approach has an added advantage of identifying clones which express viral antigens that may be most relevant to diagnostics and therapeutics. The sequences of such clones can be used to re-screen the library and "walk-out" the remaining sequences in the library; to express the encoded viral antigens by recombinant DNA techniques; or to design and synthesize peptide antigens. The antigens so produced can be used for diagnostics and/or therapeutics as described in more detail, infra.

5.2. PRODUCTION OF VIRAL PROTEINS AND ANTIGENS

In order to express HIV-LP proteins, or polypeptides or peptides derived from the viral proteins, the appropriate nucleotide sequence coding for such HIV-LP gene products, e.g. either the entire open reading frame (ORF) of an HIV-LP gene or a desired portion thereof, or a functional equivalent (hereinafter referred to as the "HIV-LP coding sequence") is inserted into an appropriate expression vector, i.e., a vector which contains the necessary elements for the transcription and translation of the inserted coding sequence. Due to the degeneracy of the genetic code, other DNA sequences which encode the same

or functionally equivalent HIV-LP gene product(s), or portion(s) thereof, may be used for cloning and expression of viral proteins and antigens. Such sequences include those which are capable of hybridizing to the HIV-LP sequence under stringent conditions, or which would be capable of hybridizing under stringent conditions but for the degeneracy of the genetic code. Such altered DNA sequences may include deletions, additions or substitutions of different nucleotide residues resulting in a sequence that encodes the same or a functionally equivalent HIV-LP gene product. Functionally equivalent gene products are those which contain deletions, additions or substitutions of amino acid residues within the HIV-LP sequence which result in a silent change.

The HIV-LP expression products as well as host cells or cell lines transfected or transformed with such recombinant expression vectors can be used for a variety of purposes. These include but are not limited to producing viral polypeptides useful in diagnostic immunoassays, vaccines, etc.

5.2.1. EXPRESSION SYSTEMS

Methods which are well known to those skilled in the art can be used to construct expression vectors containing HIV-LP coding sequences and appropriate transcriptional/translational control signals. These methods include *in vitro* recombinant DNA techniques, synthetic techniques and *in vivo* recombination/genetic recombination. See, for example, the techniques described in Maniatis et al., 1989, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y.; and Ausubel et al., 1989, Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley Interscience, N.Y.

A variety of host-expression vector systems may be utilized to express the HIV-LP coding sequences. These include but are not limited to microorganisms such as bacteria transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing the HIV-LP coding sequence; yeast transformed with recombinant yeast expression vectors containing the HIV-LP coding sequence; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing the HIV-LP coding sequence; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing the HIV-LP coding sequence; or animal cell systems infected with recombinant virus expression vectors (e.g., adenovirus, vaccinia virus) including cell lines engineered to contain multiple copies of the HIV-LP coding sequences either stably amplified (e.g., CHO/dhfr) or unstably amplified in double-minute chromosomes (e.g., murine cell lines).

The expression elements of these systems vary in their strength and specificities. Depending on the host/vector system utilized, any of a number of suitable transcription and translation elements, including constitutive and inducible promoters, may be used in the expression vector. For example, when cloning in bacterial systems, inducible promoters such as pL of bacteriophage λ, plac, ptrp, ptac (ptrp-lac hybrid promoter) and the like may be used; when cloning in insect cell systems, promoters such as the baculovirus polyhedrin promoter may be used; when cloning in plant cell systems, promoters derived from the genome of plant cells (e.g., heat shock promoters;

the promoter for the small subunit of RUBISCO; the promoter for the chlorophyll a/b binding protein) or from plant viruses (e.g., the 35S RNA promoter of CaMV; the coat protein promoter of TMV) may be used; when cloning in mammalian cell systems, promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K promoter) may be used; when generating cell lines that contain multiple copies of the HIV-LP coding sequence, SV40-, BPV- and EBV-based vectors may be used with an appropriate selectable marker.

In bacterial systems a number of expression vectors may be advantageously selected depending upon the use intended for the HIV-LP coding sequences expressed. For example, when large quantities of HIV-LP polypeptides are to be produced for the generation of antibodies, vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include but are not limited to the E. coli expression vector pUR278 (Ruther et al., 1983, EMBO J. 2:1791), in which the HIV-LP coding sequence may be ligated into the vector in frame with the lac Z coding region so that a hybrid AS-lac Z protein is produced; pIN vectors (Inouye & Inouye, 1985, Nucleic acids Res. 13:3101-3109; Van Heeke & Schuster, 1989, J. Biol. Chem. 264:5503-5509); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that

the cloned polypeptide of interest can be released from the GST moiety.

In yeast, a number of vectors containing constitutive or inducible promoters may be used. For a review see, Current Protocols in Molecular Biology, Vol. 2, 1988, Ed. Ausubel et al., Greene Publish. Assoc. & Wiley Interscience, Ch. 13; Grant et al., 1987, Expression and Secretion Vectors for Yeast, in Methods in Enzymology, Eds. Wu & Grossman, 1987, Acad. Press, N.Y., Vol. 153, pp. 516-544; Glover, 1986, DNA Cloning, Vol. II, IRL Press, Wash., D.C., Ch. 3; and Bitter, 1987, Heterologous Gene Expression in Yeast, Methods in Enzymology, Eds. Berger & Kimmel, Acad. Press, N.Y., Vol. 152, pp. 673-684; and The Molecular Biology of the Yeast Saccharomyces, 1982, Eds. Strathern et al., Cold Spring Harbor Press, Vols. I and II.

In cases where plant expression vectors are used, the expression of the HIV-LP coding sequence may be driven by any of a number of promoters. For example, viral promoters such as the 35S RNA and 19S RNA promoters of CaMV (Brisson et al., 1984, Nature 310:511-514), or the coat protein promoter of TMV (Takamatsu et al., 1987, EMBO J. 6:307-311) may be used; alternatively, plant promoters such as the small subunit of RUBISCO (Coruzzi et al., 1984, EMBO J. 3:1671-1680; Broglie et al., 1984, Science 224:838-843); or heat shock promoters, e.g., soybean hsp17.5-E or hsp17.3-B (Gurley et al., 1986, Mol. Cell. Biol. 6:559-565) may be used. These constructs can be introduced into plant cells using Ti plasmids, Ri plasmids, plant virus vectors, direct DNA transformation, microinjection, electroporation, etc. For reviews of such techniques see, for example, Weissbach & Weissbach, 1988, Methods for Plant

Molecular Biology, Academic Press, NY, Section VIII, pp. 421-463; and Grierson & Corey, 1988, Plant Molecular Biology, 2d Ed., Blackie, London, Ch. 7-9.

An alternative expression system which could be used to express HIV-LP coding sequences is an insect system. In one such system, Autographa californica nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in Spodoptera frugiperda cells. The HIV-LP coding sequence may be cloned into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter). Successful insertion of the HIV-LP coding sequence will result in inactivation of the polyhedrin gene and production of non-occluded recombinant virus (i.e., virus lacking the proteinaceous coat coded for by the polyhedrin gene). These recombinant viruses are then used to infect Spodoptera frugiperda cells in which the inserted gene is expressed. (E.g., see Smith et al., 1983, J. Viol. 46:584; Smith, U.S. Patent No. 4,215,051).

In mammalian host cells, a number of viral based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the HIV-LP coding sequence may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by in vitro or in vivo recombination. Insertion in a non-essential region of the viral genome (e.g., region E4 or E3) will result in a recombinant virus that is viable and capable of expressing the HIV-LP coding sequence in infected hosts. (E.g., See Logan & Shenk, 1984, Proc. Natl. Acad. Sci. (USA) 81:3655-3659). Alternatively, the vaccinia 7.5K promoter may

be operatively linked to the HIV-LP coding sequence which is inserted within a nonessential gene of vaccinia virus, e.g. thymidine kinase. The chimeric gene may be inserted into the vaccinia virus genome by in vivo recombination. (E.g., see Mackett et al., 1982, Proc. Natl. Acad. Sci. (USA) 79:7415-7419; Mackett et al., 1984, J. Virol. 49:857-864; Panicali et al., 1982, Proc. Natl. Acad. Sci. 79:4927-4931).

Specific initiation signals may also be required for efficient translation of inserted HIV-LP coding sequences. These signals include the ATG initiation codon and adjacent sequences. In cases where an entire HIV-LP gene sequence, including its own initiation codon and adjacent sequences, is inserted into the appropriate expression vector, no additional translational control signals may be needed. However, in cases where only a portion of an HIV-LP gene sequence is inserted, exogenous translational control signals, including the ATG initiation codon, may be provided. Furthermore, the initiation codon must be in phase with the reading frame of the HIV-LP coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and sythetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (see Bitter et al., 1987, Methods in Enzymol. 153:516-544).

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may be important for the function

of certain proteins of HIV-LP e.g., structural proteins or enzymes. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins. Appropriate cells lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such mammalian host cells include but are not limited to CHO, VERO, BHK, HeLa, COS, MDCK, 293, WI38, etc.

For long-term, high-yield production of recombinant proteins, stable expression may be preferred. For example, cell lines which stably express an HIV-LP coding sequence may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with the HIV-LP coding sequence controlled by appropriate expression control elements (e.g., promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Strong eukaryotic promoters are preferred, including but not limited to the cytomegalovirus immediate early promoter. Following the introduction of foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines.

A number of selection systems may be used, including but not limited to the herpes simplex virus

thymidine kinase (Wigler, et al., 1977, Cell 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, Proc. Natl. Acad. Sci. USA 48:2026), and adenine phosphoribosyltransferase (Lowy, et al., 1980, Cell 22:817) genes can be employed in tk$^-$, hgprt$^-$ or aprt$^-$ cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for dhfr, which confers resistance to methotrexate (Wigler, et al., 1980, Natl. Acad. Sci. USA 77:3567; O'Hare, et al., 1981, Proc. Natl. Acad. Sci. USA 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981), Proc. Natl. Acad. Sci. USA 78:2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, et al., 1981, J. Mol. Biol. 150:1); and hygro, which confers resistance to hygromycin (Santerre, et al., 1984, Gene 30:147) genes. Recently, additional selectable genes have been described, namely trpB, which allows cells to utilize indole in place of tryptophan; hisD, which allows cells to utilize histinol in place of histidine (Hartman & Mulligan, 1988, Proc. Natl. Acad. Sci. USA 85:8047); and ODC (ornithine decarboxylase) which confers resistance to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue L., 1987, In: Current Communications in Molecular Biology, Cold Spring Harbor Laboratory ed.).

The host cells which contain the coding sequence and which express the HIV-LP gene product may be identified by at least four general approaches; (a) DNA-DNA or DNA-RNA hybridization; (b) the presence or absence of "marker" gene functions; (c) assessing the level of transcription as measured by the expression of HIV-LP mRNA transcripts in the host cell; and (d) detection of the gene product as measured by immunoassay or by its biological activity.

In the first approach, the presence of the HIV-LP coding sequence inserted in the expression vector can be detected by DNA-DNA or DNA-RNA hybridization using probes comprising nucleotide sequences that are complementary to the HIV-LP coding sequence, respectively, or portions or derivatives thereof.

In the second approach, the recombinant expression vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (e.g., thymidine kinase activity, resistance to antibiotics, resistance to methotrexate, transformation phenotype, occlusion body formation in baculovirus, etc.). For example, if the HIV-LP coding sequence is inserted within a marker gene sequence of the vector, e.g. β-galactosidase, recombinants containing the HIV-LP coding sequence can be identified by the absence of the marker gene function. Alternatively, a marker gene can be placed in tandem with the HIV-LP sequence under the control of the same or different promoter used to control the expression of the HIV-LP coding sequence. Expression of the marker in response to induction or selection indicates expression of the HIV-LP coding sequence.

In the third approach, transcriptional activity for the HIV-LP coding sequence can be assessed by hybridization assays. For example, RNA can be isolated and analyzed by Northern blot using a probe complementary to the HIV-LP coding sequence or particular portions thereof.

In the fourth approach, the expression of the HIV-LP polypeptide product can be assessed immunologically, for example by Western blots, immunoassays such as radioimmuno-precipitation, enzyme-linked immunoassays and the like.

Once a clone that produces high levels of the HIV-LP polypeptide product is identified, the clone may be expanded and used to produce large amounts of the product which may be purified using techniques well-known in the art including, but not limited to immunoaffinity purification, chromatographic methods including high performance liquid chromatography, affinity chromatography.

Where the HIV-LP coding sequence is engineered to encode a cleavable fusion protein, purification may be readily accomplished using affinity purification techniques. For example, a collagenase cleavage recognition consensus sequence may be engineered between the carboxy terminus of the HIV-LP amino acid sequence and protein A. The resulting fusion protein may be readily purified using an IgG column that binds the protein A moiety. The HIV-LP product may be readily released from the column by treatment with collagenase. Another example would be the use of pGEX vectors that express foreign polypeptides as fusion proteins with glutathionine S-transferase (GST). The fusion protein may be engineered with either thrombin or factor Xa cleavage sites between the cloned gene and the GST moiety. The fusion protein may be easily purified from cell extracts by adsorption to glutathione agarose beads followed by elution in the presence of glutathione. In this aspect of the invention, any cleavage site or enzyme cleavage substrate may be engineered between the GnRH-R sequence and a second peptide or protein that has a binding partner which could be used for purification, e.g., any antigen for which an immunoaffinity column can be prepared.

5.2.2. SYNTHETIC PEPTIDES

In an alternative embodiment of the invention, the HIV-LP protein, polypeptide or peptide itself could be produced using chemical methods to synthesize the desired HIV-LP amino acid sequence in whole or in part. For example, peptides can be synthesized by solid phase techniques, cleaved from the solid phase resin, and purified by preparative high performance liquid chromatography. (E.g., see Creighton, 1983, Proteins Structures and Molecular Principles, W.H. Freeman and Co., N.Y., pp. 50-60). The composition of the synthetic peptides may be confirmed by amino acid analysis or sequencing (e.g., the Edman degradation procedure; see Creighton, 1983, Proteins, Structures and Molecular Principles, W.H. Freeman and Co., N.Y., pp. 34-49).

5.3. GENERATION OF ANTIBODIES TO HIV-LP

Various procedures known in the art may be used for the production of antibodies to epitopes of HIV-LP using whole virus, disrupted virus, isolated viral antigens, the recombinantly or synthetically produced HIV-LP proteins, polypeptides or peptides as the immunogen. Neutralizing antibodies, i.e., those which would neutralize infectivity of native HIV-LP or production of progeny virus, are especially preferred for therapeutics. Neutralizing antibodies, or antibodies which define HIV-LP serological markers would be preferred for diagnostic uses. Such antibodies may be generated using appropriate viral antigens, (e.g., identified by screening expression clones with AIDS or ARC patient antisera), as immunogens. For example, clones which express antigens that immunoreact with patient antisera generated against authentic HIV-LP may be identified in this fashion. Polypeptides or peptides produced by these

clones, or synthetic derivatives, could be used to generate appropriate antibodies that define serological markers. Antibodies which may be used in accordance with the invention include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments and fragments produced by an Fab expression library.

For the production of antibodies, various host animals may be immunized by injection with the HIV-LP protein, polypeptide, or peptide (either as a fusion protein or unfused) including but not limited to rabbits, mice, rats, etc. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and Corynebacterium parvum.

Monoclonal antibodies to HIV-LP proteins, polypeptides, or peptides may be prepared by using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include but are not limited to the hybridoma technique originally described by Kohler and Milstein, (Nature, 1975, 256:495-497), the human B-cell hybridoma technique (Kosbor et al., 1983, Immunology Today, 4:72; Cote et al., 1983, Proc. Natl. Acad. Sci., 80:2026-2030) and the EBV-hybridoma technique (Cole et al., 1985, Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). In addition, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, Proc. Natl. Acad. Sci.,

81:6851-6855; Neuberger et al., 1984, Nature, 312:604-608; Takeda et al., 1985, Nature, 314:452-454) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. Alternatively, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce HIV-LP-specific single chain antibodies.

Antibody fragments which contain the antigen-binding sites for HIV-LP may be generated by known techniques. For example, such fragments include but are not limited to: the $F(ab')_2$ fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragments. Alternatively, Fab expression libraries may be constructed (Huse et al., 1989, Science, 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity to HIV-LP.

5.4. DIAGNOSTIC ASSAYS FOR HIV-LP

The HIV-LP, and proteins, polypeptides or peptides that correspond to viral antigens, and/or antibodies to such HIV-LP antigens may be used in diagnostic immunoassays to detect HIV-LP infection. Alternatively, the HIV-LP nucleotide sequence can be used to design oligonucleotide probes/primers for use in hybridization/amplification assays for HIV-LP. The invention encompasses both the methods and kits which can be used for the detection of any strain or non-HIV-1,-2 functional equivalent of HIV-LP.

It should be understood that a certain amount of cross reactivity of HIV-LP with HIV-1 or -2 is to be expected when conducting these assays. By way of background, although HIV-2 is related to HIV-1 based on its morphology, tropism for CD4+ cells and cytopathic effects, the two viruses are distinct. Nonetheless, a certain amount of sequence similarity and serological cross-reactivity is observed between HIV-1 and -2. Generally, the cross-reactivity is restricted mostly to the major core protein, and to some extent, the envelope protein. Although HIV-LP is distinct from HIV-1 and -2, it is still a member of the HIV group of viruses, and as such, a certain similar level of cross-reactivity is to be expected between HIV-LP and HIV-1,-2.

5.4.1. IMMUNOASSAYS FOR DETECTION OF HIV-LP INFECTION

Immunoassays can be designed using HIV-LP antigens to detect patient antibodies to HIV-LP. Alternatively, the HIV-LP antigens can be used to generate antibodies (polyclonal, monoclonal, chimeric, Fab fragments, etc.) that, in turn, can be used to detect HIV-LP in infected patients.

The immunoassay kits can be designed using any label in any of a number of formats, including but not limited to, enzyme-linked immunoassays (ELISA), radioimmunoassays, and fluorescence immunoassays, which can be configured in heterogeneous or homogeneous systems, a sandwich, competitive, or displacement format, including the use of immunoprecipitation, Western blot analysis and immunoblot assays to name but a few. The immunoassay kits can be conveniently designed to test a patient's body fluids, such as serum or saliva in vitro; e.g., using a microtiter well format or an immobilized antigen-bead format. Alternatively, the immunoassay can be designed to test biopsied tissue samples, such as liver, kidney, lung, etc.

Disrupted HIV-LP may be used as a mixture of viral antigens for detecting serum antibodies, or for generating antibodies that can be used to detect HIV-LP. Alternatively, isolated viral antigens, or combinations of individual antigens, may be used. These can be produced by recombinant DNA techniques, by chemical synthetic methods, or can be isolated from disrupted HIV-LP.

Viral antigens for use in the immunoassays and kits may be identified and selected by screening an expression library with AIDS/ARC HIV-1,-2 negative patient sera, as described in Section 5.1.2, supra. In this way the coding regions for viral serological markers may be readily identified. Alternatively, the viral antigens may be identified, selected and designed by aligning the HIV-LP genome with those of HIV-1 and HIV-2; e.g., the genomes should be aligned with respect to the various HIV genes such as pol, gag, env etc., and the sequences should be configured so as to maximize homology (e.g., by introducing gaps, etc.).

Stretches of amino acids within the HIV-LP sequence corresponding to the location of antigenic regions or amino acid stretches of HIV-1 or HIV-2 which are currently used in immunoassays for HIV (e.g., in commercially available kits) may be produced by recombinant DNA or chemical synthetic techniques and used in immunoassay kits for the detection of HIV-LP.

In general, the antigenic regions of the HIV-LP polypeptides utilized can be very small, typically 7 to 10 amino acids in length. Fragments as few as 3 to 5 amino acids may characterize an antigenic region. Segments of HIV-LP polypeptides can be expressed recombinantly either as fusion proteins or as isolated polypeptides. Alternatively, short peptides can be synthetically produced. However, larger peptides, polypeptides or the entire viral protein may be utilized.

5.4.2. OLIGONUCLEOTIDE PROBES/PRIMERS FOR HYBRIDIZATION OR AMPLIFICATION ASSAYS

Oligonucleotide probes/primers can be designed on the basis of the HIV-LP sequence obtained as described herein, for use in hybridization/amplification (e.g. PCR, LCR) assays for HIV-LP. Such assay systems can be designed for detecting HIV-LP nucleic acids (i.e., virus or proviral sequences) in any of a variety of patient samples, including but not limited to PBLs (peripheral blood lymphocytes), PBMCs, tissue biopsy samples, etc.

While virtually any oligonucleotide sequence corresponding to any region of the HIV-LP genome may be utilized, in order to minimize potential cross reactivities with other HIV viruses, such as HIV-1,-2, it may be preferred to design the oligonucleotide probes/primers on the basis of sequences found in

nonconserved regions of the HIV genome; i.e., regions of the HIV-LP which are most divergent from the corresponding sequence of HIV-1,-2. Since genes such as gag and pol are relatively conserved within an HIV family, oligonucleotides corresponding to non-conserved regions of such gene sequences would be useful for distinguishing HIV-LP from other HIVs such as HIV-1 and -2. However, oligonucleotides corresponding to non-conserved regions of env, which is highly variable from strain to strain, may be preferred for identifying and distinguishing different strains of virus within the HIV-LP family.

The degree of cross-reactivity can also be controlled by the stringency of the hybridization conditions used. For example, the temperature, formamide or salt concentrations at which the annealing reactions are carried out may be increased to minimize the chances of cross-reactivity. The conditions will vary depending upon the primer used. For an example of high and low stringency conditions that can be used in PCR reaction with certain primers described herein refer to the description for FIG. 5 supra. For hybridization assays, the stringency of the washes may be controlled, as for example, explained in the description of FIG. 6, supra.

For a review of methods and conditions which can be utilized in such hybridization/amplification assay systems, see Maniatis, supra and Ausubel, supra.

5.4.3. REVERSE TRANSCRIPTASE ASSAYS

HIV-LP infection can also be diagnosed by assaying for reverse transcriptase activity in primary cultures of virus obtained from a patient using procedures well known to those skilled in the art; e.g., see Kacian, 1977, Methods In Virol. 6: 143;

Prasad & Goff, 1990, Ann. N.Y. Acad. Sci. 616: 11-21. A reverse transcriptase assay will be less specific, in that other non-HIV-LP viruses, including HIV-1 or -2 will be detected. However, the reaction conditions may be adjusted to increase the specificity of the reaction conditions for HIV-LP. For example, it is well known that the majority of retroviral polymerases preferentially use Mg^{++}, including HIV-1, which utilizes Mg^{++} much more effectively than Mn^{++} with most template primers (Hoffman et al., 1985, Virology 147: 326). However, using poly(rA)•oligo(dT) as a template, HIV-1 appears to be distinct in that it prefers Mn^{++} over Mg^{++} (Hoffman et al., supra). By contrast to HIV-1, when using the poly(rA)•oligo(dT) template, the reverse transcriptase of HIV-LP exhibits a five-fold preference for Mg^{++} over Mn^{++} as the divalent cation used in the reaction. In this regard, we have found that HIV-LP appears to prefer Mg^{++} with all artificial templates tested to date. Accordingly, in designing a reverse transcriptase assay specific for HIV-LP reaction conditions could be adjusted to include Mg^{++} or to use Mg^{++} exclusively as the cation in the reaction, e.g., using oligo(rA)•oligo(dT) template.

Alternatively, the reverse transcriptase assay can be performed on a sample in parallel, with and without neutralizing antibody specific for HIV-LP reverse transcriptase, i.e., an antibody which binds to and neutralizes the activity of the reverse transcriptase of HIV-LP but not that of HIV-1 or -2. Inhibition of the enzyme in the presence of such antibody would indicate that the reverse transcriptase activity detected is HIV-LP in origin. Likewise, antibodies that specifically neutralize HIV-1, or -2 reverse transcriptase could be used. Such antibodies

should fail to inhibit HIV-LP enzyme activity in the assay.

5.5. DEVELOPMENT OF VACCINES

A number of approaches, described in the subsections below, are possible for formulating vaccines for HIV-LP. A number of methods may be used to introduce the vaccine formulations described below, including but not limited to intravenous, oral, intradermal, intramuscular, intraperitoneal, subcutaneous, and intranasal routes. The choice, dosage, and frequency of inoculation will depend on the formulation used; e.g., live virus formulations are preferably administered via the natural route of infection; inactivated virus formulations generally require higher doses and more frequent boosts.

Since, in general, the object of immunization is to protect against disease, vaccine formulations may be designed to generate an immune response that "neutralizes" the activity of any viral antigen involved at any stage of viral replication, and is not restricted to those involved in viral binding to target cells and infection. For example, vaccines designed to generate an immune response against the HIV-LP reverse transcriptase may be as effective, if not more effective than one designed to generate an immune response against HIV-LP envelope antigens. Multivalent vaccines which incorporate two or more viral antigens may be preferred.

5.5.1. INACTIVATED VACCINES

Inactivated ("killed") vaccines may be made from the HIV-LP virus by destroying its infectivity while retaining its immunogenicity. Being non-infectious, such vaccines should be safe, but generally

need to be injected in large amounts to elicit an antibody response commensurate with that attainable by a much smaller dose of live attenuated virus. Normally, even the primary course comprises two or three injections, and further ("booster") doses may be required at intervals over succeeding years to revive waning immunity.

Purified virus is the most preferred starting material for such vaccines. Stocks of virus purified by end-dilution or cloning can be prepared as a source for vaccine formulations. For example, a single HIV-LP can be selected by serial-dilution cloning using any characteristic feature of viral infection, e.g., syncytia formation, RT activity, etc., to assay the serially diluted cultures. Alternatively, molecular biological techniques could be used to transfect T-cell lines, e.g., HUT-78, so that virus is generated from molecular clones of the integrated provirus. The virus propagated by the engineered cell can be cloned, and isolates used to prepare the viral stocks which can be used to formulate vaccines.

To prepare the large number of virions required for inactivated vaccine formulation, the purified HIV-LP virus may be propagated in cultures of mitogen-stimulated PBLs, or in cell lines that are infectable with HIV-LP and can sustain HIV-LP replication; e.g., HUT-78. The infected cells or cell lines may be grown in large volumes in suspension, in monolayers, or on microcarrier beads in fermentors. Virus may be purified and concentrated from the culture by any of a number of standard techniques including zonal ultracentrifugation, gel filtration, ion exchange chromatography, and affinity chromatography using monoclonal antibodies or a combination of such procedures. It is important to remove aggregated virus

prior to chemical inactivation to avoid contamination by residual live virus.

The most commonly used inactivating agents are formaldehyde, β-propiolactone and the ethylenimines.

5.5.2. ATTENUATED VACCINES

Live vaccine formulations may be prepared using variants of HIV-LPs that demonstrate attenuation i.e., viruses which are capable of multiplying in the host and eliciting a natural type of immune response, but which do not cause disease. Such attenuated strains may be derived from host range mutants generated by repeated passages in one or more types of cell lines which have been screened for the absence of endogenous retrovirus (e.g., host cell lines genetically engineered to express the CD4 receptor may be useful to this end); temperature-sensitive and cold-adapted mutants (although such mutants may demonstrate an unacceptable rate of reversion to wild type); or deletion mutants. In this regard, an attenuated strain may be engineered by mutagenizing HIV-LP (e.g., radiation, chemically, etc.) or site-directed mutagenesis (e.g., by deleting, adding or substituting nucleotides in viral genes which are not essential for replication, but responsible for pathogenicity). Target gene sequences of the HIV-LP which could be engineered to obtain an attenuated strain include but are not limited to nef, pol, and the transmembrane region. For example, in simian immunodeficiency virus, an amber mutation in nef resulted in reduced mortality; such mutations in HIV-LP nef may produce a similar result. Mutations in the HIV-LP polymerase which reduce its efficiency (e.g., binding affinity, enzyme activity) may reduce viral replication. Mutations in

the transmembrane region may be engineered to inhibit the cytopathic effects induced by syncytia formation. Mutations in env may alter the target cells infected and thus, result in attenuation. Care must be taken to test the non-pathogenicity of any of these engineered strains and to minimize or prevent the possibility of recombination in vivo and reversion to wild type.

5.5.3. SUBUNIT VACCINES

One or more protein(s), polypeptide(s) or peptide(s) of HIV-LP may be formulated as an immunogen in subunit vaccine formulations, which may be multivalent. Subunit vaccines comprise solely the relevant immunogenic material necessary to immunize a host. Accordingly, the relevant viral antigens of HIV-LP may be purified from virions, prepared by recombinant DNA techniques, or by chemical synthetic methods and purified described in Section 5.2 supra. With respect to recombinant DNA methods, eukaryotic host cell expression systems may be preferred for proper processing and glycosylation of HIV-LP gene products where such modification may be important; e.g. envelope. While any of the HIV-LP genes could be utilized to engineer suitable immunogens, all or portions of the polymerase (reverse transcriptase), envelope, or gag genes may be preferred.

Whether purified from virions, made by recombinant DNA technology or chemically synthesized, when isolated viral proteins, polypeptides or peptides are to be employed as vaccines, their immunogenicity can be enhanced by several orders of magnitude by coupling the protein to a suitable carrier, incorporation into a liposome, or emulsification with an adjuvant. The most widely used adjuvants in man are aluminum salts (alum), such as aluminum phosphate and

aluminum hydroxide gel. However, the resulting immune response is not particularly prolonged, therefore, booster injections are required. Other adjuvants may include, but are not limited to surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and Corynebacterium parvum.

5.5.4. RECOMBINANT LIVE VIRUS VACCINES

In an alternative approach, one or more HIV-LP gene sequences, or a desired portion(s) thereof, may be engineered into the genome of an avirulent virus that can be administered as a live recombinant vaccine. Cells in which the recombinant virus multiplies in vivo will produce the HIV-LP protein, polypeptide or peptide, against which the body will mount an immune response. For example, a recombinant live vaccinia virus which expresses one or more HIV-LP structural genes (e.g., env, gag) or viral enzymes (e.g., pol), or portions thereof, can be engineered. To this end the HIV-LP coding sequence, controlled by a strong vaccinia virus promoter (e.g., 7.5K promoter) can be inserted within a nonessential vaccinia viral gene (e.g., TK, thymidine kinase) in a plasmid. When cultured mammalian cells are infected with wild-type vaccinia and the recombinant plasmid, recombination in vivo will occur between the vaccinia DNA and the plasmid DNA resulting in the production of recombinant virions which can be purified, and expanded by propagation in cell culture. (See, e.g. Mackett et al., 1982, Proc. Natl. Acad. Sci. USA 79: 7415-7419; Mackett et al., 1984, J. Virol. 49: 857-864; Panicali et al., 1982, Proc. Natl. Acad. Sci. USA 79: 4927-4931).

6. EXAMPLE: THE ISOLATION OF A RETROVIRUS RELATED TO HIV-1 AND HIV-2 FROM PATIENTS WITH CLINICAL AIDS

The subsections below describe the isolation and characterization of a new retrovirus, related to but significantly different from HIV-1 and HIV-2, isolated from a patient with clinical AIDS. Three HIV-1,-2 seronegative individuals from New York City, two with known risk factors for acquisition of HIV, with CD4+ T-cell depletion and clinical evidence of immune deficiency consistent with Centers for Disease Control definitions of AIDS or AIDS-related complex were studied (CDC, 1987, Morbid. Mortal. Weekly Rep. 36:1S). A brief clinical description of these patients, along with three related cases is described in Section 7, infra.

6.1. CASE HISTORIES

Patient 1 (Pt. 1), the proband, is a 38 year-old white, sexually active homosexual male who presented in early 1990 with a six month history of malaise, oral candidiasis, and a cutaneous abscess. CD4+ T-cell counts ranged from 552/mm^3 to 230/mm^3 (normal 880-1677/mm^3), with CD4:CD8 ratios of 0.80 to 0.52 (normal 0.90-2.94). DNA proliferative responses of peripheral blood mononuclear cells (PBMC) to mitogen (pokeweek and phytohemagglutinin (PHA)) and antigen (tetanus toxoid) were <20% of normal controls. Serologies for HIV-1,-2 were negative by enzyme-linked immunosorbent assay (ELISA) and immunoblotting, and for human T-cell lymphotropic virus types I, II (HTLV-I, II) by immunoblotting, repeated in several labs over four years.

Patient 2 (Pt. 2) was a 73 year old Puerto Rican female whose only known risk factor for HIV was blood transfusions in 1978. She presented in late 1989

with weight loss, diarrhea, left lower extremity edema and fever, and was found to have intestinal strongyloids, Pneumocystis carinii pneumonia, Kaposi's sarcoma, and disseminated Mycobacterium tuberculosis. PBMC proliferative responses to mitogen were depressed. Serologies for HIV-1,-2 and HTLV-I/II were negative by ELISA and/or immunoblotting. The patient expired with a cerebral aneurysm.

Patient 3 (Pt. 3) was a 47 year old white heterosexual male, originally from southern Italy, who presented in mid-1990 with a clinical picture consistent with inflammatory bowel disease. He was treated with a one month course of oral glucocorticoids, which were discontinued after a diagnosis of cytomegalovirus (CMV) colitis was made by biopsy, pre-steroid therapy, of terminal ileum and cecum. Over the next three months he developed oral candidiasis, Pneumocystis carinii pneumonia, multiple Herpes simplex cutaneous infections, and a profound wasting syndrome. CD4+ T-cell counts were 76/mm^3, with concomitant depression of CD8 counts and T-cell lymphopenia. In vitro mitogen responses and serum antibody response to pneumococcal vaccination were markedly depressed. Serologies for HIV-1,-2 and HTLV-I, II were negative by ELISA and/or immunoblotting. The patient expired in mid-1991.

6.2. ISOLATION AND PRELIMINARY CHARACTERIZATION OF HIV-LP

6.2.1. PRIMARY VIRAL ISOLATES

For virus isolation, PBMCs from Pt. 1 and Pt. 2 were cultivated in the presence of PHA, or co-cultivated with equal numbers of normal donor PBMC pre-activated with PHA. One-half of the culture medium, containing interleukin-2 (IL-2), was replaced with fresh medium every 3-4 days, and monitored for reverse

transcriptase (RT) activity (Laurence et al., 1987, Science 235: 150) and HIV-1,-2 p24 Gag production. Primary cultures from Pt. 1 gave no detectable RT or p24 antigen (Ag) over a 32-day culture period (FIG. 1A), as contrasted with a representative primary culture from a known HIV-1 seropositive individual, exhibiting maximal activity, by both methods, by day 14 (FIG. 1B). PBMCs from Pts. 1 and 2 were subsequently co-cultured with PHA-activated PBMCs from two different HIV-1 seronegative healthy donors. A peak of RT activity was noted by day 28 for Pt. 1 (FIG. 1C) and day 10 for Pt. 2 (FIG. 1D). In contrast to the HIV-1+ control (FIG. 1B), and all reported retroviral isolations performed with HIV-1 or -2 seropositive individuals (Laurence et al., 1990, Cell. Immunol. 128: 337), where RT values parallel p24 Ag concentration, p24 Gag was not identified in either patient co-culture (FIG. 1C and 1D).

6.2.2. VIRAL TRANSMISSION AND CYTOPATHICITY

Both cell-free and cell-associated transmission of this RT activity was observed. In two representative experiments, 50 μl of filtered (0.2μ) supernatant from cultures at the height of RT activity were added to donor PHA-activated PBMC. RT activity was observed by day 13-17 of culture (FIG. 1E and 1F), again in the absence of Gag Ag. Similar results were obtained with Pt. 2 supernatants. Cytopathic effects typical of HIV-1 were detected in many cultures, with multinucleated giant cells (FIG. 2) and extensive cytolysis. For example, in the experiments of FIG. 1E and 1F there were 39.1 ± 5% viable cells (trypan blue vital dye exclusion method; Laurence, et al., 1990, Cell. Immunol. 128:337) in Pt. 1 supernatant-exposed

cultures on day 15, compared to 87.0 ± 3% viability in control co-cultures.

6.2.3. PURIFICATION AND MORPHOLOGIC IDENTIFICATION OF VIRUS

A simultaneous detection assay was used to segregate particulate RT activity from contaminating cellular DNA polymerases. Supernatants of Pt. 1 co-cultures were cleared by centrifugation at 1000 x g, layered over a discontinuous gradient of sucrose in TNE buffer (10 mM Tris-HCl, pH 7.4, 0.1 M NaCl, 1 mM EDTA), and centrifuged at 33,000 rpm in a SW41 rotor for 12h at 4°C, as described (Kacian, 1977, Methods Virol. 6: 143). The RNA-directed DNA polymerase activity using a poly(rA).oligo(dT)$_{12-18}$ template (P-L Biochem., Milwaukee, WI) peaked in fractions with a density between 1.12-1.16 g/ml. This is characteristic of mammalian retroviruses (Kacian, 1977, Methods Virol. 6: 143), and paralleled fractionation of an HIV-1 control supernatant.

6.2.4. CELLULAR TROPISM OF VIRUS ISOLATE

The tropism of Pt. 1 isolate was determined by its capacity to replicate in donor PBMC in the presence or absence of an anti-CD4 monoclonal antibody, Leu-3a, known to block CD4-dependent cell entry of HIV and SIV. Complete abrogation of RT activity was observed in antibody-treated cultures.

6.2.5. ELECTRON MICROSCOPY

Preliminary examination of ultra-thin sections of fixed pellets from Pt. 1 cell co-cultures by electron microscopy revealed only occasional particles of diameter 100-110nm with morphologic similarities to HIV and SIV (FIG. 4). No definitive membrane budding, intracellularly or extracellularly,

was noted, perhaps related to the low RT counts of these samples, as compared with typical co-cultures of HIV-1.

6.3. ANALYSIS OF VIRAL ANTIGENS

6.3.1. IMMUNOPRECIPITATION OF VIRAL ANTIGENS

As noted above, serum samples from all three patients failed to react by ELISA and immunoblotting with Ags from HIV-1,-2 and HTLV- I, II; this is illustrated for HIV-1 and Pts. 1 and 2 in FIG. 3A. Metabolic labeling of Pt. 1 isolated-infected PBMC with [^{35}S]cysteine, immunoprecipitation of soluble extracts from these cultures, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was then performed. Serum from Pt. 1 precipitated four bands, including molecules of molecular weight 130-140, 41 and 27 kD (FIG. 3B). By analogy to HIV-1,-2, these may represent envelope, transmembrane, and group antigen (core) structures. Serum from six of seven HIV-1 seropositive donors and one HIV-2 seropositive donor failed to recognize any unique epitopes in Pt. 1 extracts; one of the HIV-1+ subjects gave a radioimmunoprecipitation pattern similar to that of Pt. 1 serum (FIG. 3B). Pt. 2 serum similarly recognized Ags. of Pt. 1 extracts.

6.3.2. SEROLOGIC REACTIVITIES

Indirect immunofluorescence was performed with either mouse anti-human monoclonal antibodies counterstained with fluorescein-conjugated $F(ab')_2$ fragments of goat anti-mouse IgG (Tago Inc., Burlingame, CA), or with a 1:20 dilution of human sera, then counterstained with fluorescein-conjugated $F(ab')_2$ fragments of goat anti-human IgG, IgA and IgM (Cappel Laboratories, Cochranville, PA). Details of these

procedures have been previously described (Laurence et al., 1987, J. Clin. Invest. 80: 1631-9). Cells were analyzed by flow cytometry using an EPICS-V cytofluorograph.

Sera from other HIV-1 seropositive individuals, selected for varying clinical manifestations of HIV-1 disease as well as differing dates of initial diagnosis, were also examined for the ability to recognize Pt. 1-specific membrane antigens by indirect immunofluorescence assay. Sera from normal controls, an HIV-1,-2 seronegative and retrovirus culture negative individual with Pneumocystis carinii pneumonia (Patient N, Table 1), and an HIV-2 seropositive reagent (L657, Table I) failed to recognize Pt. 1 infected PBMC (Table I). However, one of 7 HIV-1 positive sera screened did react with Pt. 1 infected PBMC (Table I). An estimate of the percent infected cells, determined by cytofluorimetry with Pt. 1 serum, was approximately 9%. This is in good agreement with the frequency of infected cells typically found in HIV-1 infected PBMC cultures (Sarngardharan et al., 1984, Science 224: 506-8). The reaction noted between Pt. 1 and HIV-1 sera may be due either to cross reaction between Pt. 1 and HIV-1 sera as observed for HIV-1 and -2, or to co-infection of HIV-LP in patients infected with HIV-1. For example, in one large survey, cross-reactions against HIV-2 env glycoproteins were observed by immunoblot in 10% and by RIPA in 40% of the HIV-1 antibody positive sera (Bottiger et al., 1990, J. Virol. 64: 3492-9). Pt. 2 serum also recognized a significant proportion of Pt. 1 infected cells (Table I).

TABLE I

INDIRECT IMMUNOFLUORESCENCE ANALYSIS
OF PBMC INFECTED WITH RT+ Pt. 1 SUPERNATANT

Subject	Date Serum Obtained	HIV-1 Status	Diagnosis	IFA* (% positive cells) TARGET PHA-PBMC	PHA-PBMC/ Pt. 1
Pt. 1	4/90	-	immune deficiency	0.7	5.7
Pt. 2	11/89	-	immune deficiency Kaposi's sarcoma	2.7	8.5
Control-H	8/89	-	normal donor	0	0
S	2/87	+	AIDS		0
F	10/83	+	asymptomatic		0.7
R	2/83	+	lymphadenopathy		0.8
J	1/84	+	asymptomatic		0
R	2/84	+	asymptomatic		7.2
V	11/83	+	Kaposi's sarcoma		0
W	10/83	+	AIDS, pediatric pt.		0
N	9/87	-	Pneumocystis carinii pneumonia		0
L657	-	-	HIV-2, West African pt.		0.9

* Serum samples were used at a 1:20 dilution. Indicator cell targets were from 3 week cultures of PHA-activated normal donor PBMC (PHA-PBMC) or PBMC infected with RT+ Pt. 1 supernatants and maintained for the same culture interval, representing the peak or RT activity.

6.4. MOLECULAR CHARACTERIZATION OF THE VIRUS

6.4.1. PCR ASSAYS

To further study relationships among HIV-1,-2, Pt. 1 isolate and material from Pts. 2 and 3, DNA was extracted from fresh PBMCs (Pts. 1 and 2), paraffin-embedded lymphoid tissue (Pt. 3), and viral co-cultures (Pts. 1 and 2), then assayed for HIV-1,-2 gag and HIV-1 tat by PCR. This was first performed under conditions of stringency (temperature, [Mg++]) appropriate for each primer pair, and a "hot start" protocol capable of detecting one molecule of HIV DNA in the presence of 1μg of human DNA on an ethidium-stained gel (Mullis, 1991, PCR Methods Applications 1: 1). The gag primers amplify sequences conserved among all known HIV-1,-2 isolates, permitting up to 2 mismatches at the 3'-terminus (Kwok, et al., 1990, Nuc. Acids Res. 18:999; Ou, et al., 1988 Science 239: 295). The tat primers were of particular utility, capable of detecting HIV-1 sequences in DNA extracted from multiple PBMC and paraffin-embedded tissue specimens from HIV-1 seropositive individuals.

In addition, DNA from Pt. 1 PBMC was independently assessed by PCR for HIV-1 gag and env (Dept. of Diagnostics Research, Cetus Corp., Emmeryville, CA), and from Pt. 3 for HIV-1,-2 gag and pol (Dr. B. Poiesz, SUNY Health Science Center, Syracuse, NY). No HIV-1,-2 sequences were detected by these methods (FIG. 5). Upon lowering the stringency for primer annealing, HIV-1,-2 related gag signals were detected in all samples tested, while only Pt. 3 gave a tat amplicon of expected size (FIG. 5).

A set of degenerate primer oligonucleotides capable of detecting pol gene sequences from HIV-1,-2 and SIV, as well as other mammalian lentiviruses with no cross-reactivity with HTLV-I/II or human endogenous

retroviral sequences, was also used to probe DNA from Pts. 1 and 3. The two sets of degenerate primers utilized were: LV1, 5'-CCGGATCCDCAPyCCNGSAGGAPyTAMAA, and LV2, 5'-GGTCTAGAPyPuPyAPuTTCATAACCCAKCCA, where D=G, T or A; Py=C or T, Pu=A or G, S=C or G; M=C or A; and LV3, 5'-CCGGATCCGApyPuTPuGGKGAPyGCMTA, where K=G or T, and DDMY, 5'-CCGGATCCPuTCPuTCCTPuTA. No product of expected size (450bp) was noted on a first round of amplification under high stringency using primer pair LV1 and LV2, while second round amplification under high stringency with the nested primers LV3 and DDMY gave PCR products of anticipated size (254 bp), as recognized by ethidium bromide agarose electrophoresis.

6.4.2. DOT BLOT HYBRIDIZATION ASSAYS

Dot-blot hybridizations of HIV-1 strain TIIIB RNA and RNA from ultracentrifuged supernatants from Pt. 1 co-cultures were then performed utilizing subgenomic DNA probes under conditions of varying stringency. Strong hybridization was seen with one probe representing the entire 3' half of HIV-1 under low stringency, with a weaker signal detected using a more restricted sequence (FIG. 6A and 6B).

6.4.3. CLONING AND SEQUENCING OF VIRAL cDNA

Preliminary sequence information was obtained by cloning of complementary DNA (cDNA) from the viral RNA of Pt. 1 co-cultures (FIG. 7A, 7B and 7C), using established procedures (Clavel et al., 1986, Nature 324: 691). In one region, a 215 base stretch of *env*-like sequences gave a 71% match with HIV-1 isolate SF-2 (nucleotides 6612-6827, FIG. 7A) corresponding, at the nucleotide level, to a relatively conserved region, the gp120 V1 loop. In a second 274bp fragment there was a

40.1% match with a region of the HIV-1 long terminal repeat, including nef (FIG. 7B). In a third fragment there was a 56% match with the pol region (nucleotides 3347-3524) of HIV-1 (FIG. 7C).

To identify the putative tat amplicon from Pt. 3 PCR product, a pre-screen, utilizing the TA Cloning System (Invitrogen, San Diego, CA) was used prior to sequencing of the 142 bp product. A sequence identical to an HIV-1 tat consensus (Myers et al., eds. Human Retroviruses and AIDS 1991: a compilation and analysis of nucleic acid and amino acid sequences, Los Alamos National Lab., Los Alamos, NM, 1991), except for 5 nucleotide changes (T for A at position 56, A for G at 62, A for C at 88, C for G at 147 and G for C at position 149), resulting in five amino acid alterations, was obtained. Finally, as an additional assessment of this isolate's position among lentiviruses, codon usage was obtained for all sequences derived to date. High A, low C content is restricted to mammalian lenti- and spumaviruses (Myers & Pavlakis, In J. Levy, ed. HIV, Plenum, NY, 1992, p. 72). For Pt. 1 isolate env sequence (FIG. 7A), A was 31.5% and C 18.1%, which parallels to that for HIV (35.4% A, 17.8% C for SF-2) and spumaviruses (mean 32.5% A, 19.1% C) (Myers & Pavlakis, In J. Levy, ed. HIV, Plenum, NY, 1992, p.72).

6.5. ELISAs FOR HIV-LP

Based on these PCR and sequence data, a search for HIV Pol reactive antibodies in Pt. 1 and 2 sera was conducted using an epitope scanning kit (Cambridge Research Biochemicals, Wilmington, DE) containing decapeptides spanning amino acid residues 144-536 of HIV-1 clone HXB2 (Myers et al., eds. Human Retroviruses and AIDS 1991: a compilation and analysis

of nucleic acid and amino acid sequences, Los Alamos National Lab., Los Alamos, NM, 1991), and an ELISA detection system previously described (Laurence, et al., 1984, N. Engl. J. Med. 311: 1269). Strong reactivity was found with seven decapeptides (positivity defined as ≥3 SD above reactivity with normal sera), including residues 294-303 (PLTEEAELEL), which define a region recognized by several sera from HIV-1 seropositive individuals (Laurence et al., 1987, Science 235: 1501) as well as monoclonal antibodies prepared from HIV-1 Pol-immunized mice capable of specifically inhibiting the catalytic activity of HIV-1 Pol (Orvell, et al., 1991, J.Gen. Virol. 72: 1913). Similar techniques were applied to a preliminary ELISA screen of various sera with SDS lysates of ultracentrifuges pellets from supernatants of Pt. 1 infected cultures. Strong reactivity was obtained with sera from Pts. 1 and 2, as well as one of ten HIV-1 seropositive individuals at various clinical stages of infection (optical density > 1.5 at serum dilutions of 1:100, with backgrounds from donor serum <0.2). This latter result may reflect co-infection of the individual with both HIV-1 and our novel HIV isolate. Such assays will be facilitated by use of Pt. 1 isolate propagated in continuous cell lines and, indeed, low-level RT activity has been identified in HUT-78 CD4+ T-cells inoculated with this virus.

7. EXAMPLE: CLINICAL DESCRIPTION OF AIDS PATIENTS INFECTED WITH HIV-LP

Five individuals from the New York City area, four with known risk factors for human immunodeficiency virus (HIV) infection, with profound CD4+ T-cell depletion and clinical syndromes consistent with definitions of AIDS-related complex or AIDS (CDC, 1987, MMWR, 1987; 36: 1S-14S) are described. All lacked

evidence of HIV-1,-2 infection, as assessed by multiple serologies over several years, standard viral co-cultures for HIV p24 Gag antigen, and proviral DNA amplification by polymerase chain reaction. Standard serologies were performed for HIV-1 by ELISA and immunoblotting, for HIV-2 by immunoblotting or competition peptide ELISA, and for human T-cell lymphotropic virus types, I, II (HTLV-I, II) by immunoblotting. Viral isolations were attempted using peripheral blood mononuclear cells (PBMC) co-cultured with phytohemagglutinin-activated normal donor PBMC in the presence of interleukin-2, with evidence for HIV activity sought by ELISA-based assay for HIV-1,-2 p24 Gag in culture supernatants. Further evidence for HIV was sought by PCR-directed DNA amplification, using primers as outlined in each case.

Patients 1, 2 and 3 are described in Section 6, supra.

Patient 4 is a 37 year-old white male health care worker with a history of multiple heterosexual partners. Over the past two years he has suffered intractable cutaneous papillomavirus infections, Molluscum contagiosum, and Herpes zoster. CD4+ T-cell counts have fluctuated from 120-200/mm^3, with poor in vitro responses to T and B cell mitogens, and anergy to several antigens. Serologies for HIV-1,-2 and PCR for HIV-1,-2 gag have been negative.

Patient 5 is a 35 year-old Hispanic, sexually active homosexual male who presented with a three month history of chronic cough and dyspnea. Conversion of PPD skin test positivity was documented, and Mycobacterium tuberculosis identified on thoracentesis of an extensive pleural effusion. A CD4+ T-cell count was 289/mm^3. Repetitive HIV-1 ELISAs, and PCR for HIV-1,-2 gag sequences have been negative.

These five cases, as well as two in the literature (Safai et al., Kaposi's sarcoma among HIV seronegative high-risk populations. VII Intl. Conf. AIDS, florence, Italy, June 16-21, 1991, Abst. TuB83; Castro, et al., 1992, Lancet, 339: 868), illustrate individuals with acquired cellular immune deficiencies and clinical case histories consistent with AIDS or ARC, in the absence of evidence for known retroviral infection by serology, PCR-directed DNA amplification, and standard viral cultures. Given risk factors for spread of HIV in many of these individuals, these cases raise the question of the existence of other agents lined to transmissible immune deficiencies which can evade current laboratory detection techniques.

8. DEPOSIT OF MICROORGANISMS

The following microorganisms have been deposited with the American Type Culture Collection, (ATCC), Rockville, Maryland and have been assigned the following accession numbers:

Microorganism	Date of Deposit	Accession No.
HIV-LP	June 12, 1992	VR 2374
JS-2	June 12, 1992	69012
JS-8	June 12, 1992	69013
JS-5	June 12, 1992	69011
JS-3	June 18, 1992	69018

The present invention is not to be limited in scope by the microorganisms deposited since the deposited embodiments are intended as illustrations of single aspects of the invention and any microorganisms which are functionally equivalent are within the scope of the invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying

drawings. Such modifications are intended to fall within the scope of the appended claims.

It is also to be understood that all base pair sizes given for nucleotides are approximate and are used for purposes of description.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Gelman, Irwin H.
Laurence, Jeffrey C.

(ii) TITLE OF INVENTION: A Novel Human Immunodeficiency Virus

(iii) NUMBER OF SEQUENCES: 3

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Pennie & Edmonds
(B) STREET: 1155 Avenue of the Americas
(C) CITY: New York
(D) STATE: New York
(E) COUNTRY: U.S.A.
(F) ZIP: 10036-2711

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Misrock, S. Leslie
(B) REGISTRATION NUMBER: 18,872
(C) REFERENCE/DOCKET NUMBER: 6923-023

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 212 790-9090
(B) TELEFAX: 212 869-8864/9741
(C) TELEX: 66141 PENNIE

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 215 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AAGCTTAGAG TCTTGGATCT AGTGACATTC TTATATTTGC TTTCCTTTAT ATTGTGGTAT 60

ATTTTTGAGC TTAATTATTA AACATAAATA CTCATCAAGG TCAAGGATCT GAAATCCCAT 120

TCAGAAAGAA AATGCAACAA TTGGAACCCT GTGCAACCTA GAAGACATTG GGCACTGAAT 180

AAAGTGGATT TCCAGGAGCT CTCCGTTTGC AACTC 215

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 274 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TGGTTGCGTG GCTCATGCCT GTAATCTAAG CACTTTGGGA GGCCAAGGTG GGAAGATTGC 60

TTGAGCCCAG TAGTTGGAGA CCAGGCTAGG CAACGTGGAG AGACCCAATC TCTACAAAAA 120

TTTTAAAAAT GAGCTGAGTG TGGTAGATCA CGACTGTGGC CCTGCTACTC TGGAGGCCGA 180

GGCAAGAGGA TTCCCTGAGC TCAGGAGGTT GAGGCTCGAC TGAGCCATGA TCACACCACT 240

GCACTCCAGC CTGGCAACAG GTAGAGCCAT GTTT 274

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 169 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGTAGGACAG GCAACAGACT ACAAAGGAAA CATAAAGTAA GGAATCCCCC AGGGACTAGA 60

CAAAGGGAAA TTACCAAAAG AAAATAAATA GGATAAGGAA AATAAGAAAA GAAATCAACC 120

TTTTGATTCA TCGGAAAAAA GTACAAAAAA AAACCAAAAT CCAGGATTC 169

International Application No: PCT/ /

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 49, lines 17-36 and page 50, lines 1-2 of the description *

A. IDENTIFICATION OF DEPOSIT *

Further deposits are identified on an additional sheet *

Name of depositary institution *

American Type Culture Collection

Address of depositary institution (including postal code and country) *

12301 Parklawn Drive
Rockville, MD 10582
US

Date of deposit * June 18, 1992 Accession Number * 69018

B. ADDITIONAL INDICATIONS * (Leave blank if not applicable). This information is continued on a separate attached sheet ☒

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE * (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS * (Leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later * (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☐ This sheet was received with the International application when filed (to be checked by the receiving Office)

David N. Russell
(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau *

was

(Authorized Officer)

Form PCT/RO/134 (January 1981)

International Application No: PCT/ /

Form PCT/RO/134 (cont.)

American Type Culture Collection

12301 Parklawn Drive
Rockville, MD 10582
US

Accession No.	Date of Deposit
69011	June 12, 1992
69012	June 12, 1992
69013	June 12, 1992
VR 2374	June 12, 1992

WHAT IS CLAIMED IS:

1. A purified HIV-LP virus.

2. A non HIV-1,-2 human retrovirus that immunologically cross-reacts with an antibody to the HIV-LP isolate as deposited with the ATCC and assigned accession no. VR 2374.

3. A cultured cell infected with HIV-LP.

4. A continuous cell line infected with HIV-LP.

5. An isolated HIV-LP polynucleotide, the sequence of which corresponds to the HIV-LP genome or provirus.

6. The isolated polynucleotide of Claim 5 which comprises RNA.

7. The isolated polynucleotide of Claim 5 which comprises DNA.

8. An oligonucleotide having a sequence complementary to a portion of the HIV-LP genome or provirus.

9. An oligonucleotide which is capable of hybridizing under stringent conditions to a portion of the HIV-LP genome or provirus.

10. A recombinant DNA vector containing a nucleotide sequence that encodes an HIV-LP gene product or an epitope thereof.

11. A recombinant DNA vector containing a nucleotide sequence that encodes a fusion protein comprising an HIV-LP gene product or an epitope thereof fused to a heterologous polypeptide.

12. The recombinant DNA vector of Claim 10 in which the nucleotide sequence that encodes the HIV-LP gene product or epitope thereof is operatively associated with a regulatory sequence that controls gene expression in a host.

13. The recombinant DNA vector of Claim 11 in which the nucleotide sequence that encodes the fusion protein is operatively associated with a regulatory sequence that controls gene expression in a host.

14. The recombinant DNA vector of Claim 10, 11, 12 or 13 in which the HIV-LP gene product is the envelope protein.

15. The recombinant DNA vector of Claim 10, 11, 12 or 13 in which the HIV-LP gene product is the transmembrane protein.

16. The recombinant DNA vector of Claim 10, 11, 12 or 13 in which the HIV-LP gene product is the core protein.

17. The recombinant DNA vector of Claim 10, 11, 12 or 13 in which the HIV-LP gene product is the reverse transcriptase.

18. The recombinant DNA vector of Claim 10, 11, 12 or 13 which is capable of hybridizing under

stringent conditions, or which would be capable of hybridizing under stringent conditions but for the degeneracy of the genetic code to the HIV-LP env gene.

19. The recombinant DNA vector of Claim 10, 11, 12 or 13 which is capable of hybridizing under stringent conditions, or which would be capable of hybridizing under stringent conditions but for the degeneracy of the genetic code to the HIV-LP gag gene.

20. The recombinant DNA vector of Claim 10, 11, 12 or 13 which is capable of hybridizing under stringent conditions, or which would be capable of hybridizing under stringent conditions but for the degeneracy of the genetic code to the HIV-LP pol gene.

21. A host cell transformed with the recombinant DNA vector of Claim 10, 11, 12, or 13.

22. A method for producing an HIV-LP gene product or epitope thereof, comprising:

(a) culturing a host cell transformed with the recombinant DNA expression vector of Claim 12 and which expresses the HIV-LP gene product or epitope thereof; and
(b) recovering the expressed product from the cell culture.

23. A method for producing fusion protein comprising an HIV-LP gene product, or epitope thereof, fused to a heterologous peptide, comprising:

(a) culturing a host cell transformed with the recombinant DNA expression

vector of Claim 13, and which expresses the fusion protein; and

(b) recovering the fusion protein from the cell culture.

24. The method of Claim 22 or 23 in which the HIV-LP gene product is the envelope protein.

25. The method of Claim 22 or 23 in which the HIV-LP gene product is the transmembrane protein.

26. The method of Claim 22 or 23 in which the HIV-LP gene product is the core protein.

27. The method of Claim 22 or 23 in which the HIV-LP gene product is the reverse transcriptase.

28. The method of Claim 22 or 23 in which the recombinant DNA vector is capable of hybridizing under stringent conditions, or would be capable of hybridizing under stringent conditions but for the degeneracy of the genetic code, to the HIV-LP env gene.

29. The method of Claim 22 or 23 in which the recombinant DNA vector is capable of hybridizing under stringent conditions, or would be capable of hybridizing under stringent conditions but for the degeneracy of the genetic code, to the HIV-LP gag gene.

30. The method of Claim 22 or 23 in which the recombinant DNA vector is capable of hybridizing under stringent conditions, or would be capable of hybridizing under stringent conditions but for the degeneracy of the genetic code, to the HIV-LP pol gene.

31. A peptide, polypeptide or protein encoded by the genome of HIV-LP.

32. The peptide, polypeptide or protein of Claim 31 which contains an epitope of HIV-LP.

33. The peptide, polypeptide or protein of Claim 31 that immunologically cross reacts with an antibody to the HIV-LP isolate as deposited with the ATCC and assigned accession no. VR 2374.

34. The peptide, polypeptide or protein of Claim 31, 32 or 33 having an amino acid sequence homologous to the envelope protein of HIV-LP.

35. The peptide, polypeptide or protein of Claim 31, 32 or 33 having an amino acid sequence homologous to the transmembrane protein of HIV-LP.

36. The peptide, polypeptide or protein of Claim 31, 32 or 33 having an amino acid sequence homologous to the core protein of HIV-LP.

37. The peptide, polypeptide or protein of Claim 31, 32 or 33 having an amino acid sequence homologous to the reverse transcriptase of HIV-LP.

38. A fusion protein comprising an HIV-LP gene product, or an epitope thereof, fused to a heterologous polypeptide.

39. The fusion protein of Claim 38 that immunologically cross reacts with an antibody to the HIV-LP isolate as deposited with the ATCC and assigned accession no. VR 2374.

40. The fusion protein of Claim 38 in which the HIV-LP gene product has an amino acid sequence homologous to the envelope protein of HIV-LP.

41. The fusion protein of Claim 38 in which the HIV-LP gene product has an amino acid sequence homologous to the transmembrane protein of HIV-LP.

42. The fusion protein of Claim 38 in which the HIV-LP gene product has an amino acid sequence homologous to the core protein of HIV-LP.

43. The fusion protein of Claim 38 in which the HIV-LP gene product has an amino acid sequence homologous to the reverse transcriptase protein of HIV-LP.

44. An isolated antibody that immunospecifically binds to an epitope of HIV-LP.

45. An isolated antibody that immunospecifically binds to a serological marker of HIV-LP.

46. The antibody of Claim 44 or 45 which is polyclonal.

47. The antibody of Claim 44 or 45 which is monoclonal.

48. A method for detecting HIV-LP nucleic acids in a sample, comprising

(a) reacting nucleic acids of the sample with an oligonucleotide probe for HIV-LP under conditions

which allow the formation of a polynucleotide duplex between the probe and HIV-LP nucleic acid in the sample; and

(b) detecting a polynucleotide duplex which contains the probe as an indication of the presence of HIV-LP nucleic acids in the sample.

49. The method according to Claim 48 in which the HIV-LP nucleic acid in the sample is amplified.

50. A kit for analyzing sample for the presence of nucleic acids derived from HIV-LP comprising an oligonucleotide probe of Claim 8 or 9 in a suitable container.

51. The kit of Claim 50 which additionally contains a polymerase to amplify the HIV-LP nucleic acids.

52. An immunoassay for HIV-LP antigen, in a sample, comprising

(a) contacting the sample with an antibody that immunospecifically binds to an epitope of HIV-LP under conditions which allow formation of antibody-antigen complex; and

(b) detecting antibody-antigen complex in the sample as an indication of the presence of HIV-LP antigen in the sample.

53. An immunoassay for antibodies to HIV-LP in a sample, comprising

(a) contacting the sample with a peptide, polypeptide, protein or fusion protein containing an HIV-LP epitope under conditions which allow formation of antibody-antigen complex; and

(b) detecting antibody-antigen complex in the sample as an indication of the presence of antibody to HIV-LP in the sample.

54. The immunoassay of Claim 52 or 53 in which the HIV-LP epitope is an envelope epitope.

55. The immunoassay of Claim 52 or 53 in which the HIV-LP epitope is a transmembrane epitope.

56. The immunoassay of Claim 52 or 53 in which the HIV-LP epitope is a core protein epitope.

57. The immunoassay of Claim 52 or 53 in which the HIV-LP epitope is a reverse transcriptase epitope.

58. An immunoassay kit for analyzing samples for the presence of HIV-LP antigen comprising an antibody that immunospecifically binds to an epitope of HIV-LP, in a suitable container.

59. An immunoassay kit for analyzing samples for the presence of antibodies to HIV-LP, comprising a peptide, polypeptide, protein or fusion protein

containing an epitope of HIV-LP, in a suitable container.

60. An HIV-LP, vaccine formulation, comprising an immunogenically effective dose of inactivated HIV-LP in a pharmaceutically acceptable carrier.

61. An HIV-LP vaccine formulation, comprising an immunologically effective dose of attenuated HIV-LP in a pharmaceutically acceptable carrier.

62. An HIV-LP vaccine formulation, comprising an immunogenically effective dose of a peptide, polypeptide, protein or fusion protein containing an HIV-LP antigen in a pharmaceutically acceptable carrier.

63. An HIV-LP vaccine formulation comprising an immunologically effective dose of a recombinant virus which directs the expression of an HIV-LP epitope, in a pharmaceutically acceptable carrier.

FIGURE 1

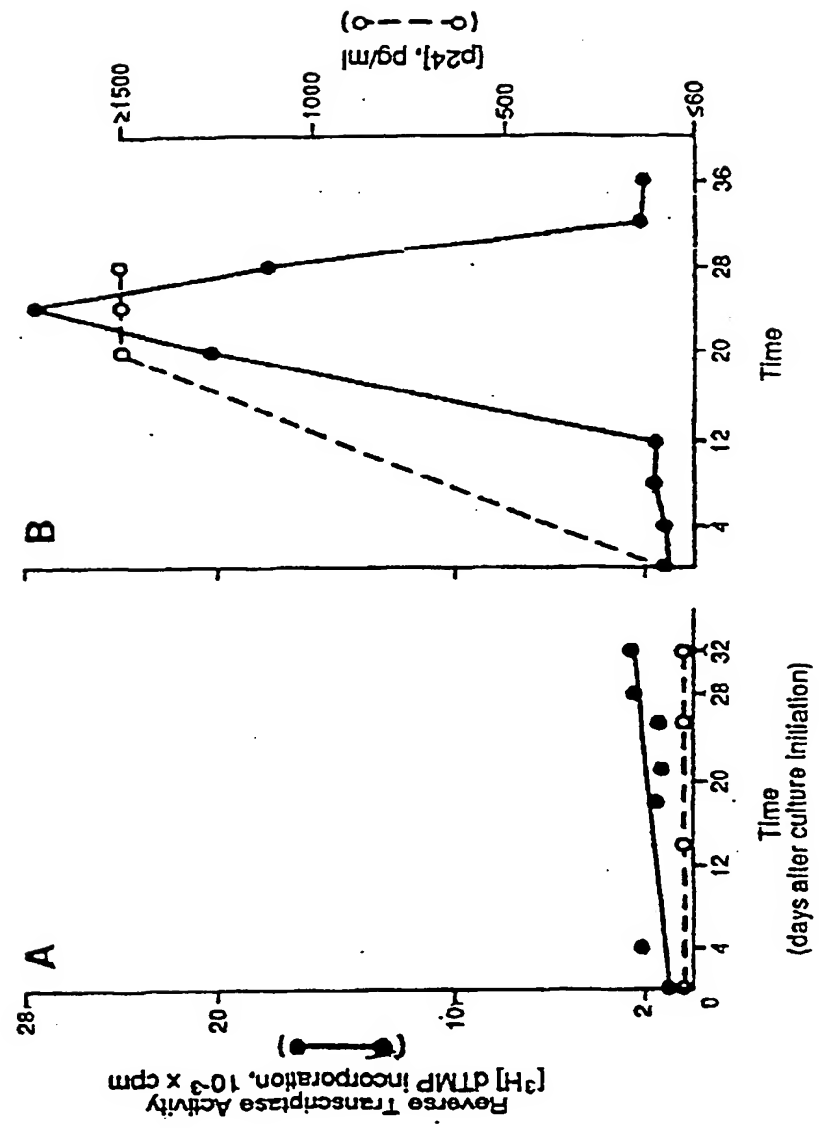
A
B
Reverse Transcriptase Activity
[3H] dTMP incorporation, 10-3 x cpm
[p24], pg/ml
Time
(days after culture initiation)
28
20
10
2
0
4
12
20
28
32
≥1500
1000
500
≤60
36

FIGURE 1 (continued)

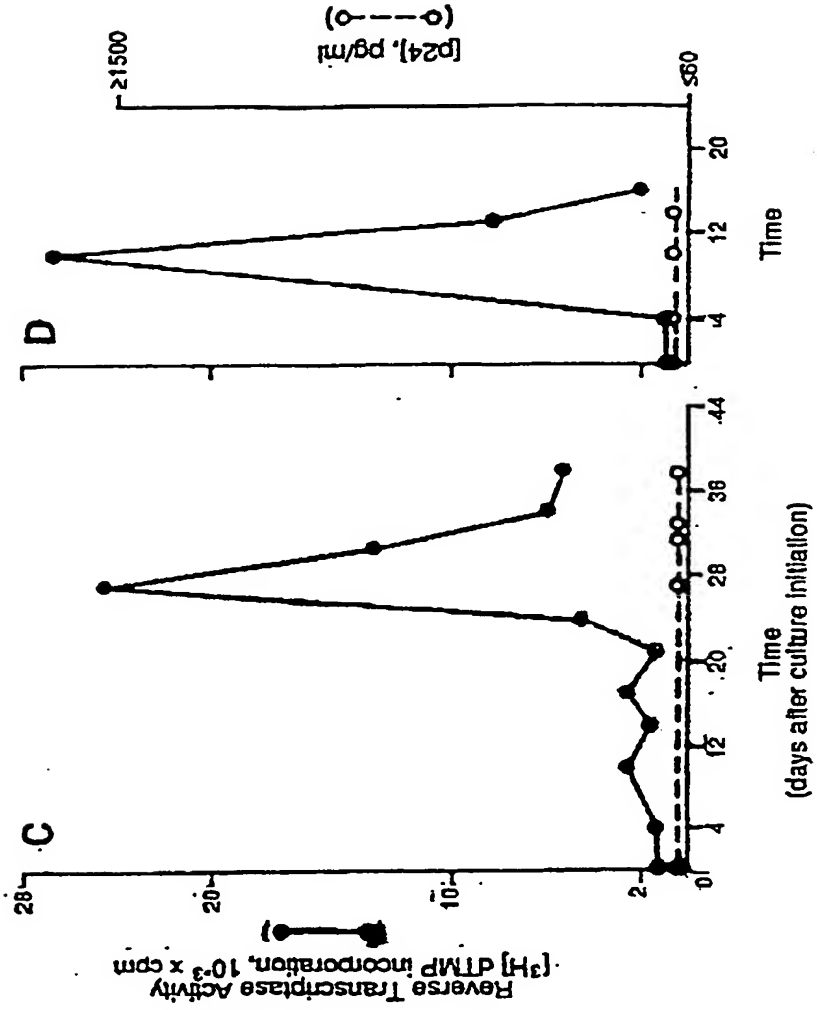

FIGURE 1 (continued)

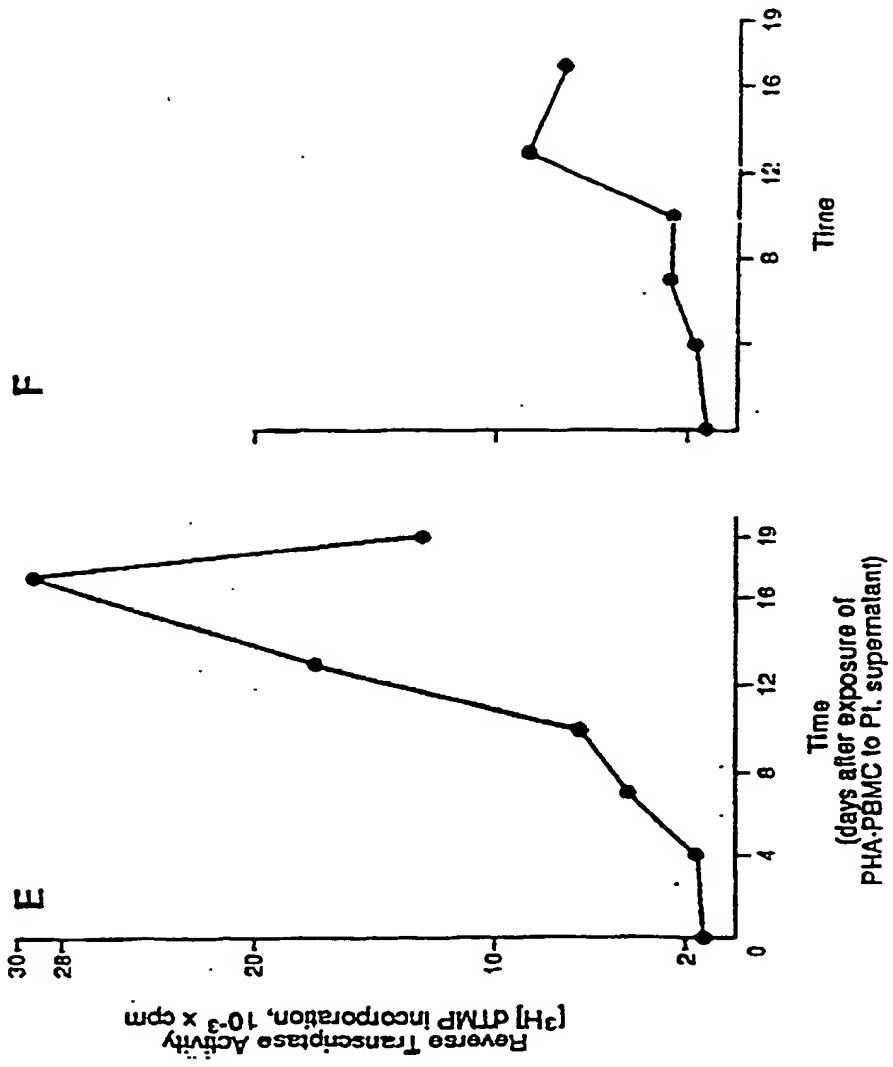

Figure 2

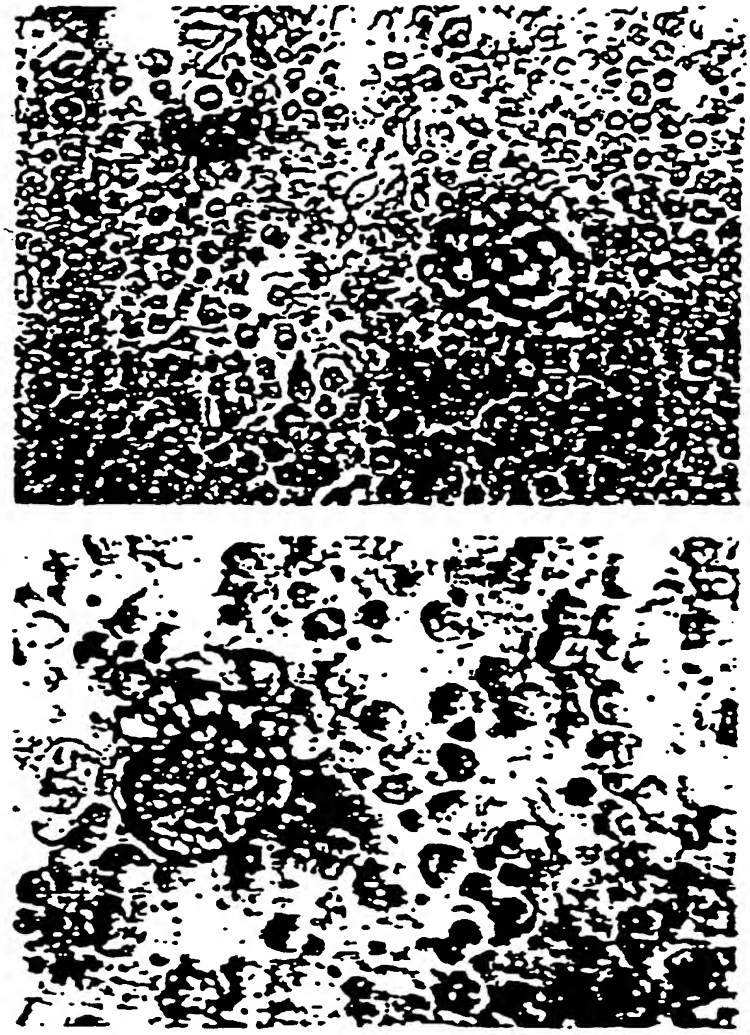

Figure 3


A
1 2 3 4
MW (kD)
160
120
55
41
24
18
B
MW (kd)
1 2 3 4 5
200
150
97
69
45
30
18
12

Figure 4

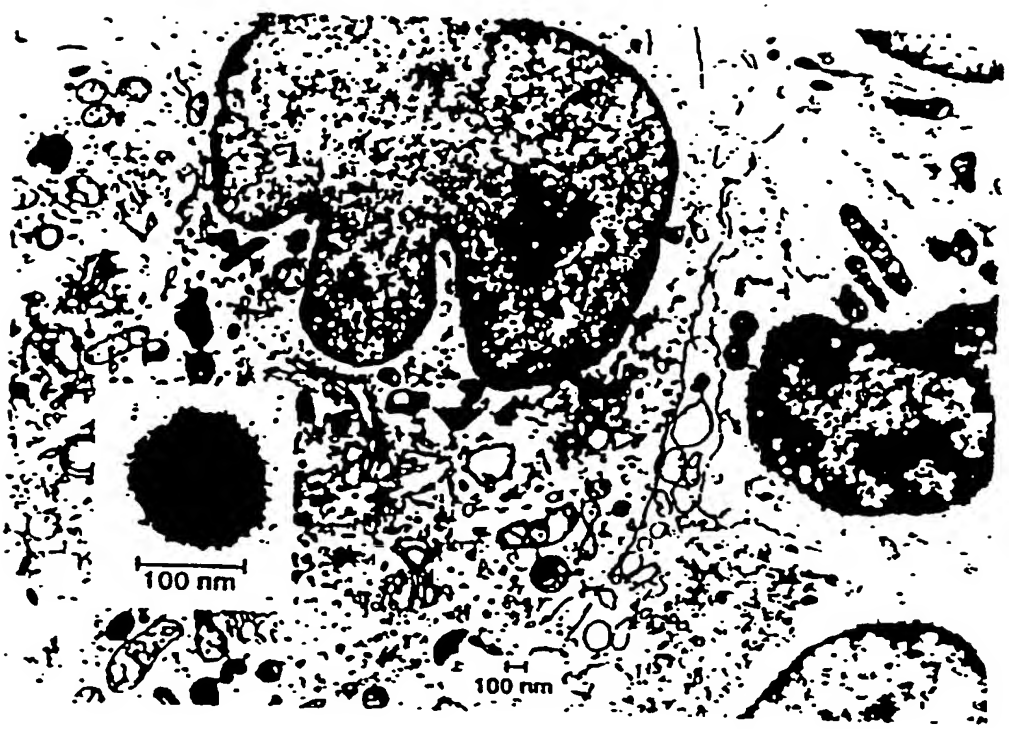

Figure 5

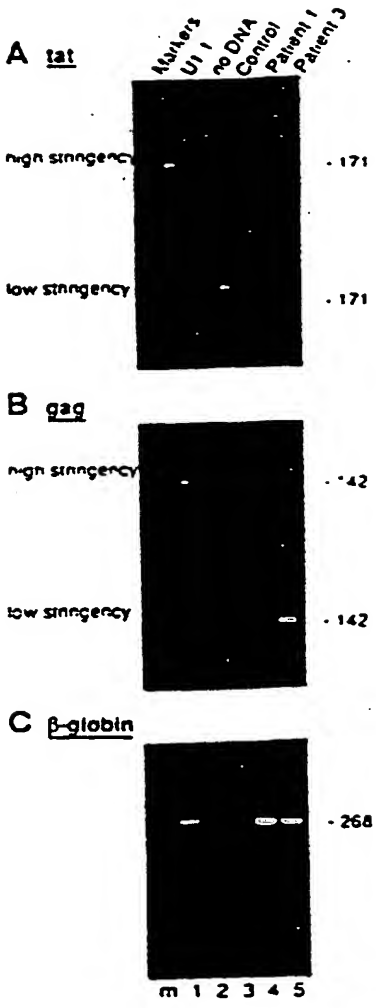
A tat
Markers
U1 1
no DNA Control
Patient 1
Patient 3
high stringency
low stringency
171
171
B gag
high stringency
low stringency
142
142
C β-globin
268
m 1 2 3 4 5

Figure 6 (A and B)

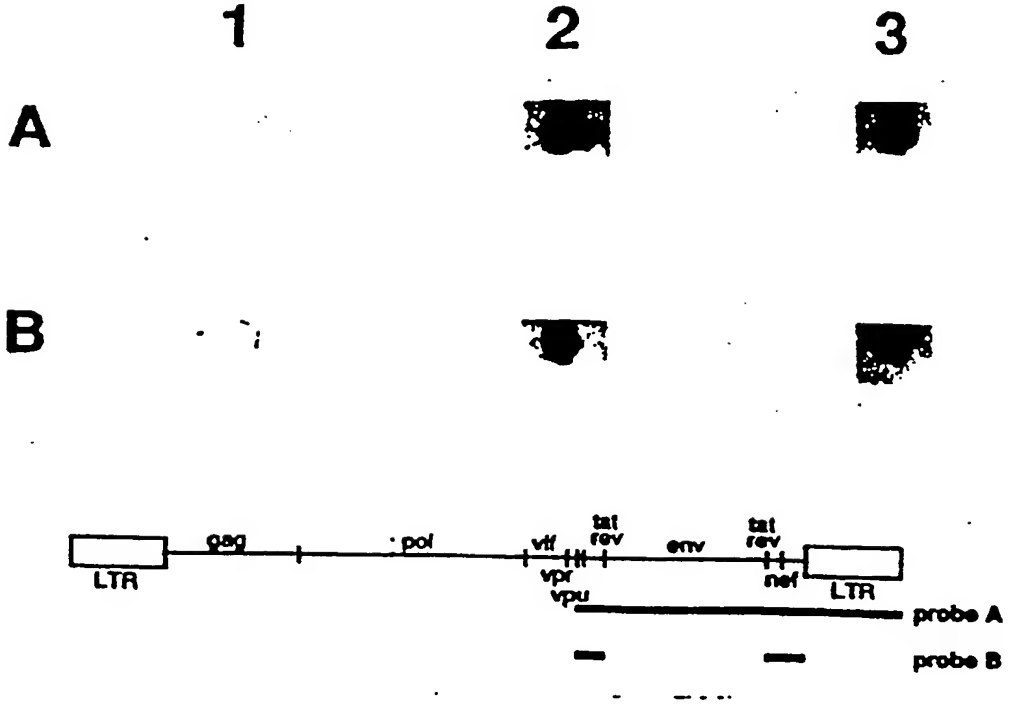

Figure 7 (A, B and C)

A

```
  1  AAGCTTAGAG TCTTGGATCT AGTGACATTC TTATATTTGC TTTCCTTTAT
 51  ATTGTGGTAT ATTTTTGAGC TTAATTATTA AACATAAATA CTCATCAAGG
101  TCAAGGATCT GAAATCCCAT TCAGAAAGAA AATGCAACAA TTGGAACCCT
151  GTGCAACCTA GAAGACATTG GGCACTGAAT AAAGTGGATT TCCAGGAGCT
201  CTCCGTTTGC AACTC
```

B

```
  1  TGGTTGCGTG GCTCATGCCT GTAATCTAAG CACTTTGGGA GGCCAAGGTG
 51  GGAAGATTGC TTGAGCCCAG TAGTTGGAGA CCAGGCTAGG CAACGTGGAG
101  AGACCCAATC TCTACAAAAA TTTTAAAAAT GAGCTGAGTG TGGTAGATCA
151  CGACTGTGGC CCTGCTACTC TGGAGGCCGA GGCAAGAGGA TTCCCTGAGC
201  TCAGGAGGTT GAGGCTCGAC TGAGCCATGA TCACACCACT GCACTCCAGC
251  CTGGCAACAG GTAGAGCCAT GTTT
```

C

```
  1  ggtaggacaggcaacagactacaaaggaaacataaagtaaggaatccccccagg
 54  g...actagacaaagggaaattaccaaaaganaaataaataggataaggaaaaat
104  aagaaaagaaatcaaccttttgattcatcggaaaaaagtacaaaaaaaaccca
154  aaatccaggattc
```

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/06162

A. CLASSIFICATION OF SUBJECT MATTER
IPC(5) :C12N 7/00; C12Q 1/68, 1/70; C07K 15/02, 15/12; A01N 63/00
US CL :435/5, 6,235; 530/350, 387; 424/93
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/5, 6, 235; 530/350, 387; 424/93

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NATURE, Vol. 330, issued 12 November 1987, Hahn et al, "Relation of HTLV-4 to simian and human immunodeficiency-associated viruses", pages 184-186, see entire document.	1-4 & 60-61
X	THE LANCET, issued 06 December 1986, Biberfeld et al, "Findings in Four HTLV-IV Seropositive Women from West Africa", pages 1330-1331, see entire document.	1-4 & 60-61
X	SCIENCE, Vol. 236, issued 15 May 1987, Kanki et al, "Human T-Lymphotropic Virus type 4 and the Human Immunodeficiency Virus in West Africa", pages 827-831, see entire document.	1-4 & 60-61

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be part of particular relevance
"E" earlier document published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 September 1993	24 SEP 1993
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer CHRISTINE NUCKER Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/06162

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NATURE, Vol. 342, issued 21/28 December 1989, Dietrich et al, "A highly divergent HIV-2 related isolate", pages 948-950, see entire document.	1-4 & 60-61

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/06162

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
(Telephone Practice)

I. Claims 1-4 and 60-61, drawn to a purified virus and infected cells.
II. Claims 5-30, drawn to nucleic acid.
III. Claims 31-59 and 62, drawn to viral protein, antiviral antibodies and methods of use for the protein and antibodies.
IV. Claims 63, drawn to a vaccine with a recombinant virus.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-4 and 60-61

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.